Centro de Investigación Científica de Yucatán, A.C.

Posgrado en Ciencias Biológicas

CARACTERIZACIÓN MOLECULAR DE GENES QUE MODULAN LA TRANSCRIPCIÓN Y EL TRANSPORTE DE AUXINAS Y SU PAPEL EN LA RIZOGÉNESIS EN VITROPLANTAS DE *Carica papaya* L. cv. MARADOL

Tesis que presenta

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RECONOCIMIENTO

Por medio de la presente, hago constar que el trabajo de tesis de **Humberto José Estrella Maldonado** titulado "**Caracterización molecular de genes que modulan la transcripción y el transporte de auxinas y su papel en la rizogénesis en vitroplantas de *Carica papaya* L. cv. Maradol**", fue realizado en la Unidad de Biotecnología, en la línea de Agrobiotecnología, en el laboratorio de Fisiología Vegetal y Molecular de papaya del Centro de Investigación Científica de Yucatán, A.C. bajo la dirección del Dr. Jorge M. Santamaría Fernández, dentro de la Opción Biotecnología, perteneciente al Programa de Posgrado en Ciencias Biológicas de este Centro.

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Este trabajo se llevó a cabo en la Unidad de Biotecnología del Centro de Investigación Científica de Yucatán, y forma parte del proyecto titulado Factores de transcripción en sequía, eficiencia en el uso de agua. Clave: 6020700003 en el que participé bajo la dirección del Dr. Jorge M. Santamaría Fernández.

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RESUMEN

Las plantas de algunas especies incluyendo *C. papaya*, cuando son cultivadas *in vitro* presentan una eficiencia intermedia de enraizamiento. La formación de raíces, requiere la acción coordinada de genes que regulan a células meristemáticas y aquellos que regulan el transporte celular, ambos sistemas son inducidos por auxinas.

La señalización de auxinas es controlada por factores de transcripción (FTs) del tipo ARFs (factores de respuesta a auxina) y AUX/IAA (represores de la transcripción en respuesta a auxina), estos FTs activan o reprimen procesos de formación de raíces laterales. Por otra parte, se han definido genes que modulan el transporte polar de auxina (PAT) de tipo AUX1/LAX y PIN, los cuales participan en el desarrollo y formación de raíces.

El objetivo del presente trabajo radica en comprender mecanismos moleculares de la rizogénesis mediante posibles cambios de expresión génica de FTs (ARFs y Aux/IAA) y de PAT (AUX/LAX y PIN) en vitroplantas de *C. papaya* L. cv. Maradol expuestas a diferentes sistemas inductores de la rizogénesis. Los resultados indican que las vitroplantas expuestas a IBA exógena adicionado al medio de cultivo, fueron capaces de formar raíces, y como resultado presentaron altas tasas de sobrevivencia *ex vitro* (93 %). En forma interesante, estas vitroplantas presentaron baja expresión de genes represores CpAUX/IAAs y alta expresión de genes activadores CpARFs. De igual forma dichas vitroplantas tratadas con IBA presentaron alta expresión de CpAUX1/LAXs y CpPINs en tejidos de la base del tallo y raíces, mientras que lo contrario ocurrió en tratamientos donde las vitroplantas no fueron capaces de producir raíces.

ABSTRACT

Plants of some species, including *C. papaya*, when cultivated *in vitro*, have an intermediate rooting efficiency. Root formation requires the coordinated action of genes that regulate meristematic cells and those that regulate cell transport, both auxin-induced systems.

Auxin signaling is controlled by transcription factors (TFs) of the type ARFs (auxin response factors) and AUX/IAA (transcription repressors of response to auxin), these FTs activate or repress lateral root formation processes. On the other hand, it has been established that genes that modulate auxin polar transport (PAT) of the type AUX1/LAX and PIN, are involved in the development and formation of roots.

The objective of the present work is to understand the molecular mechanisms of rhizogenesis, via possible gene expression changes of FTs (ARFs and Aux/IAA) and PAT (AUX/LAX and PIN) on *in vitro* plantlets of *C. papaya* L. cv. Maradol exposed to different rhizogenesis-inducing systems. The results indicate that *in vitro* plantlets exposed to exogenous IBA added to the culture medium, are able to form roots, and as a result they had high *ex vitro* survival rates (93 %). Interestingly, these *in vitro* plantlets presented low expression of CpAUX/IAAs repressor genes but high expression of CpARFs that is an auxin activator system. In addition, those IBA treated plants had high expression levels of the CpAUX1/LAXs and CpPINs genes in stem base and root tissues, whereas the opposite occurred in treatments where *in vitro* plantlets were unable to produce roots.

INTRODUCCIÓN

Carica papaya L. pertenece a la familia Caricaceae. Es una planta arborescente tropical originaria de Mesoamérica y ampliamente cultivada en las zonas tropicales del continente americano (Anandan et al., 2011). Esta especie crece en regiones tropicales y subtropicales de América y en países africanos (Cronquist, 1981). A nivel mundial, el fruto de *C. papaya* es de alto consumo y su demanda se ha incrementado no sólo por presentar buenas características sensoriales y nutritivas, sino también por el beneficio que aportan ciertas biomoléculas como son los carotenoides (Yamamoto, 1964).

Por lo anterior, es necesario crear protocolos de micropropagación mediante técnicas de cultivo *in vitro* con la finalidad de reproducir masivamente plantas de *C. papaya* con características deseables de producción, resistentes a enfermedades y que las plantas obtenidas presenten buenos porcentajes de enraizamiento para su correspondiente trasplante en campo. Sin embargo, la propagación *in vitro* de *C. papaya* L. revela que los principales inconvenientes para propagar esta especie son el bajo enraizamiento *ex vitro* que deriva en mortandad de las plantas durante su aclimatación en invernadero (Gangopadhyay et al., 2009; Anandan et al., 2011). Actualmente, el principal objetivo de los propagadores para minimizar la mortandad de las vitroplantas de *C. papaya* es que las plantas regeneradas forman formen un buen sistema radical bajo condiciones *in vitro* y *ex vitro*, debido a que su nutrición dependerá durante mucho tiempo y en gran medida de la funcionalidad de sus raíces, con este objetivo se han usado fitohormonas como las auxinas, las cuales han promovido la inducción de raíces adventicias en especies como *Zea mays (Z. mays)*, *Arabidopsis thaliana* (*A. thaliana*) y *Oriza sativa (O. sativa)* entre otras especies (Drew et al., 1979; Wample and Reid, 1979).

Durante mucho tiempo la auxina, ha sido ampliamente utilizada por promover la formación de raíces laterales y adventicias (Torrey, 1950; Laskowski et al., 1995; Blakely et al., 1988). En *A. thaliana*, las raíces laterales son iniciados por la activación local auxínico de las células del periciclo en los polos del xilema (Casimiro et al., 2001; 2003), sin embargo, los detalles moleculares de la acción de la auxina en procesos rizogénicos permanecen en gran medida desconocidos (Okushima et al., 2007), pero su estudio es un requisito necesario para comprender como la auxina modula funciones durante la regulación de su

biosíntesis, transporte y la capacidad de las células para responder de una manera apropiada (Okushima et al., 2007).

Aunque es poca la información de la cascada de señalización de la auxina durante la rizogénesis, el genoma secuenciado de *C. papaya* L. cv. Sun Up (Ming et al., 2008) nos brinda la ventaja de realizar búsquedas para poder identificar y caracterizar genes involucrados en la regulación y transporte de auxina. Con esta posibilidad de identificar genes, en nuestro trabajo de investigación describimos genes transportadores de auxinas de flujo de entrada (CpAUX1/LAXs) y de salida (CpPINs) los cuales son necesarios para distribuir diferencialmente la auxina de célula a célula mediante un transporte polar de auxina (PAT), este transporte de auxina regula el mecanismo de iniciación y desarrollo de las raíces laterales (Dubrovsky et al., 2008). Además describimos factores de transcripción (FTs) de auxina los cuales regulan mecanismos involucrados en la formación radical de la planta. En este contexto se han descrito en *C. papaya* dos familias de genes relacionados con la respuesta a la auxina, el primero pertenece a la familia de genes inducidos por auxina (CpAux/IAA) y el segundo a factores de respuesta a auxina (CpARFs).

En el presente estudio se utilizaron herramientas bioinformáticos para identificar en el genoma secuenciado de papaya transgénica Sun Up genes involucrados en el transporte polar de auxina de entrada (CpAUX1, CpLAX1, CpLAX2, CpLAX3) y de salida (CpPIN1, CpPIN2, CpPIN3, CpPIN4, CpPIN5 y CpPIN6) y de genes reguladores de la transcripción de auxinas (CpARF5, CpARF6, CpARF7 y CpAux/IAA12, Cp Aux/IAA13 y Cp Aux/IAA14). Posteriormente sus patrones de expresión fueron evaluados en diferentes tratamientos rizogénicos con y sin la presencia de auxina involucrada en el enraizamiento como es el ácido indol butírico (IBA) en la base del tallo y en raíces de vitroplantas de *C. papaya* cv. Maradol. El interés final de este trabajo radica en comprender los mecanismos moleculares de la rizogénesis mediante los posibles cambios de expresión de genes activadores (CpARFs) y represores (CpAux/IAA) de la transcripción de la auxina y como se correlacionan con los cambios de expresión de genes involucrados al transporte polar de auxina (CpAUX1/LAXs - CpPINs) en vitroplantas *C. papaya* L. cv. Maradol.

CAPÍTULO I.

ANTECEDENTES GENERALES

1.1. ANTECEDENTES

1.1.1 Origen y distribución de *Carica papaya* L.

Carica papaya L. (*C. papaya*) es originaria de las planicies de la región centroamericana; su producción se ha extendido en mayor medida a países tropicales y subtropicales del mundo; su lugar de origen exacto se desconoce, aunque diferentes autores señalan a México y Centroamérica como países de origen (Fuentes and Santamaría, 2014). En la actualidad es cultivada en todas las regiones tropicales de América, desde México hasta Brasil, y en muchas regiones del mundo. En México, *C. papaya* se distribuye alrededor del Golfo de México, desde Tamaulipas hasta la Península de Yucatán y por el Pacífico se le encuentra desde Baja California hasta Chiapas. Se localiza desde el nivel del mar hasta los 1,500 metros sobre el nivel del mar (Anandan et al., 2011). Las diferentes variedades de *C. papaya* se han nombrado en función del tamaño, forma, apariencia y procedencia de la fruta, aunque la variedad predominante es del cultivar (cv.) Maradol. Actualmente existen sembradas 12,500 hectáreas de papaya distribuidas principalmente en 8 estados de la República Mexicana, el 80 % de la producción está en: Veracruz, Colima, Michoacán, Chiapas, Oaxaca, y Yucatán; el 20 % restante se concentra en: Jalisco, Puebla y Sinaloa (Propapaya, 2015).

1.1.2. Generalidades de *Carica papaya* L.

El cultivo de papaya representa un gran impacto socioeconómico debido a que el 90 % del fruto de papaya se destina al consumo fresco, el 10 % restante se destina a elaborar productos procesados tales como harinas. Entre las variedades más conocidas está la papaya cv. Maradol, la papaya amarilla, la roja y la mamey. El fruto necesita crecer en zonas lluviosas, con 1,800 milímetros anuales y con una humedad relativa del 85 % y temperatura de 25 ºC. Puede resistir fríos ligeros, pero no debe cultivarse en áreas donde haya heladas o temperaturas bajo cero, ya que la planta moriría (SIAP, 2012).

1.1.3. Descripción botánica de *Carica papaya* L.

La planta de *C. papaya* es vigorosa y productiva, además se adapta a gran variedad de suelos, el peso de sus frutos en estado maduro varían entre 3 a 7 Kg. Las plantas de papaya presentan un comportamiento muy característico para esta especie, las flores se presentan de la siguiente manera: los tipos I (Femenina), las de tipo II (Elongata) y por último las de tipo III (Masculina), la cual casi nunca aparece cuando se utiliza una semilla procedente de un buen programa de mantenimiento genético (Rodríguez et al., 2002).

El fruto presenta maduración lenta, pulpa suave y gran consistencia. Su piel es lisa, gruesa y resistente, presentando larga vida de anaquel. Por dentro es rojiza o anaranjada, con numerosas semillas parietales. Las semillas son de color negro, redondeado u ovoide, y están encerradas en un arillo transparente, gelatinoso. Las plantas de papaya sembradas normalmente por semilla, bajo condiciones ideales pueden germinar en dos semanas, pero pueden tardar hasta cinco semanas. Estas plantas obtenidas de semillero florecen entre nueve meses y un año después de la germinación. Los frutos se cosechan cuando la mayor parte de la piel es de color verde amarillo. Después de varios días a temperatura ambiente, los frutos alcanzaran la madurez de consumo, presentando una coloración completamente amarillas y suaves al tacto (Rodríguez et al., 2002).

La planta de papaya cv. Maradol produce frutos hermafroditas (cilíndricos y alargados) y frutos femeninos (redondos), de color rojo salmón en su interior al madurar y de color naranja brillante en su exterior cuando alcanza la madurez fisiológica, estos frutos grandes y carnosos, presentan un tamaño que oscila entre 22 a 27 cm de largo y de 9 a 13 cm de diámetro, su cavidad mide entre 3 a 4.5 cm, además, presenta un promedio de 12 °Brix en relación al contenido de sólidos solubles totales, pudiendo bajar si existe carencia de potasio asimilable en el suelo (Jiménez and Rodríguez, 1998). La recolección de los frutos se realiza cuando estos empiezan a ablandarse y al perder el color verde del ápice. Debido a su piel delgada, son frutos muy delicados que se dañan fácilmente. Por ello, durante su transporte y su posterior comercialización se deben envolver uno por uno y empacarse acojinados (SIAP, 2012).

1.1.4. Clasificación taxonómica de *Carica papaya* L.

La papaya es una planta dicotiledónea que pertenece a la familia Caricaceae, una familia que contiene 35 especies agrupados en cuatro géneros (*Carica, Cylicomorpha, Jarilla* y *Jacaratia*), los híbridos pertenecientes a esta familia evidencian una considerable variabilidad genética que constituye una fuente importante para el mejoramiento vegetal, la más importante es la de tipo *Carica*, la cual presenta usos agrícolas, medicinales e industriales (Teixeira da Silva et al., 2007). Si bien existe una gran variedad de papayas, el género *Carica* es el único que se cultiva como árbol frutal mientras que los otros géneros son cultivados principalmente como ornamentales. Entre todas las especie de ese género la más común es la papaya lechosa *C. papaya* (Teixeira da Silva et al., 2007). En la Tabla 1.1 el Sistema Integrado de Información Taxonómica (ITIS, 2013), clasifica a la *C. papaya* de la siguiente manera:

Tabla 1.1. Jerarquía Taxonómica de *Carica papaya* L.

Reino	Plantae
Sub-reino	Viridaeplantae
Super-división	Embriofita
División	Traqueofita
Clase	Magnoliopsida
Orden	Brasicales
Familia	Caricaceae
Género	*Carica* L.
Especie	*Carica papaya* L.

Fuente: ITIS (última actualización: 11 de Diciembre de 2016).

1.1.5. Producción de papaya a nivel mundial

Según la FAOSTAT, hasta el año 2014 en el mundo existían 411,355 hectáreas (has) dedicadas al cultivo de papaya. El pasí de la India siembran 133,360 has de papaya, continuando Brasil (32,031 has), Indonesia (9,384 has), Nigeria (94,200 has) y México (14,533 has). La producción mundial de este cultivo ha aumentado de 7 millones de toneladas (tons) en el año 2000 hasta 12.6 millones en 2014. Para este mismo año, India tuvo una producción de 5.6 millones de tons, seguido de Brasil (1.6 millones de tons), Nigeria (850 mil tons), Indonesia (840 mil tons) y México (836 mil tons) (FAOSTAT, 2014).

1.1.6. Producción de papaya en México

México tuvo un incremento en la producción de papaya al pasar de 764,514 tons en el año 2010 a 836,370 tons en el 2014 y con respecto al área de cosecha del fruto, México sembró 14,533 has de papaya en el 2014. (FAOSTAT, 2014). Referente a las exportaciones, México es el principal país exportador de papaya hacia los Estados Unidos y Canadá (SIAP, 2015). En 2014, México exportó a los Estados Unidos la cantidad de 6,765 embarques de 40,000 lbs. equivalentes a 121 mil tons de papaya, esta cifra significó un aumento del 13 % superando lo exportado en 2009, la cantidad más alta de exportación del fruto de papaya hasta el momento registrada (Propapaya, 2015).

1.1.7. Producción de papaya en Yucatán

En Yucatán, la papaya era un cultivo tradicional, en donde las áreas plantadas de papaya criolla antes de 1995 alcanzaban unos 150 has, su producción tenía como objetivo los mercados locales y centros turísticos.

El programa de papaya Maradol en Yucatán nace a finales de 1995, considerando los antecedentes de la introducción de esta variedad en el estado durante los años 1993 y 1994 (Gobierno del Estado de Yucatán, 1999). De acuerdo con las estadísticas de Propapaya (2014), para el año 2014, Yucatán ocupó el sexto lugar produciendo el 6 % de la superficie sembrada de papaya a nivel nacional. Así mismo, el estado de Yucatán mantiene 500 has de papaya de las cuales 300 has se encuentran en producción activa del fruto. Yucatán mantiene relaciones comerciales respecto a la exportación de papaya a los Estados Unidos por medio de Miami, este fruto ha sido reportado de gran interés para su exportación al mercado Europeo.

1.1.8. Problemática de las plantas en condiciones *in vitro*

Condiciones de cultivo *in vitro* tales como baja intensidad luminosa, baja o alta temperatura, alta humedad relativa, composición de los nutrientes en el medio de crecimiento, adición de azúcares al medio de cultivo y condiciones no estériles en que se desarrollan las vitroplantas de *C. papaya* pueden causar alteraciones anatómicas y fisiológicas en las plantas regeneradas en comparación con plantas obtenidas mediante la germinación de semillas (Pospíšilová et al., 1999; Zobayed et al., 2000; Correll and Weathers, 2001; Premkumar et al., 2001; Rodríguez et al., 2003; Aragón et al., 2005). Tales alteraciones afectan la sobrevivencia o adaptación de las vitroplantas cuando son trasplantadas en invernadero (Hazarika, 2003).

Las plántulas propagadas *in vitro* poseen características anatómicas y fisiológicas diferentes de las cultivadas convencionalmente: son semi-autótrofos (Hazarika, 2003), las hojas presentan una cutícula más delgada (Majada et al., 2000) formadas durante su crecimiento debido a que no alcanzan la capacidad fotosintética deseable a causa del limitado acceso al CO_2 en el interior del recipiente donde crece (Hazarika, 2003), consecuencia de lo anterior sus estomas presentan un funcionamiento deficiente (Shackel et al., 1990), células en empalizada no uniformes (Fabbri et al., 1986; Smith et al., 1986), tejidos hiperhidratados (Majada et al., 2000) y modificación en el sistema vascular (Piccotino et al., 1997) entre otras. Todas estas condiciones fisiológicas y las características intrínsecas de las plántulas *in vitro* tienen influencia en la sobrevivencia en invernadero. Por lo tanto, se han utilizado estrategias para que plántulas producidas *in vitro* se asemejen a las propagadas convencionalmente; tal es el caso de reducir o eliminar la fuente de azúcar en el medio de cultivo (Zobayed et al., 2000), incrementar la intensidad luminosa en el cuarto de incubación (Wilson et al., 2001), elevar la concentración de CO_2 dentro de los contenedores (Heo et al., 2001), disminuir la humedad relativa dentro de los contenedores para estimular cambios significativos en el crecimiento, anatomía y fisiología de las plántulas (Nguyen et al., 2001). Una óptima regulación de tales condiciones podrían mejorar el crecimiento de las vitroplantas al transferirlas bajo condiciones *ex vitro* (Hazarika, 2003).

La suplementación exógena de sacarosa en el medio de cultivo aumenta las reservas de almidón satisfaciendo las demandas de energía suministrando una adecuada fuente de carbono para el crecimiento y diversas funciones fisiológicas de las vitroplantas, además de ayudar en el mantenimiento del potencial osmótico de las células (Pospíšilová et al., 1999). Durante el enraizamiento *in vitro*, se ha observado que la iniciación de la raíz y la posibilidad de un mejor enraizamiento puede aumentar de manera significativa al disminuir los niveles de sales minerales en el medio de cultivo e incrementando la concentración de sacarosa del 2 al 5 %, lo cual mejora la aclimatación (Pierik et al., 1975; Díaz-Pérez et al., 1995; Soukup et al., 2004).

La luz es otro factor a considerar en la aclimatación de las vitroplantas. La luz representa una señal ambiental importante que induce la fotomorfogénesis e interactúa con las señales endógenas, incluyendo hormonas. Durante la transición del crecimiento de la vitroplanta, la luz disminuye la tasa de elongación del hipocotilo, los ganchos se despliegan, los cotiledones se abren y la maquinaria fotosintética se desarrolla. Tales cambios son rápidos y complejos, por tanto, una serie de fotorreceptores están involucrados en la detección de la cantidad y calidad de la luz, incluyendo fitocromos receptores de luz (rojo [R] y rojo lejano [FR]), criptocromos receptores de luz (azul [B] y UV-), fototropinas (receptores de luz B) y fotorreceptores UV-B (Casal et al., 1998; Briggs and Olney, 2001). Una vez que los fotorreceptores se activan, las señales de luz son transferidos a las redes moleculares corriente abajo que desencadenan: crecimiento y respuestas de desarrollo, implicando fitohormonas (Kraepiel and Miginiac, 1997). Liu et al. (2011) encontraron que la luz aumenta el transporte de la auxina endógena en hipocotilos de *A. thaliana* y *Solanum lycopersicum*. Plantas de semillero de maíz (*Zea mays*) cultivadas en oscuridad, se les expuso bajo dos tratamientos: plantas expuestas en un corto tiempo a luz rojo y plantas expuestas durante varias horas a luz rojo, en ambos casos se encontró que se indujo una disminución de IAA libre y se provocó un aumento de IAA conjugado (Bandurski et al., 1977; Jones et al., 1991; Barker-Bridges et al., 1998; Zelená, 2000). Shinkle et al., (1998) encontraron que 30 hasta 50 horas continuas de luz rojo, aumentó el transporte de IAA en pepinos etiolados (*Cucumis sativus*) en segmentos de hipocotilo.

1.1.9. Problemática de las vitroplantas de papaya: enraizamiento y aclimatación

Dada la importancia económica que representa el consumo de *C. papaya* en especial del cultivar Maradol, se han establecido estrategias biotecnológicas como es la micropropagación, para la multiplicación de esta especie, sin embargo, hasta la fecha la escala comercial de papaya ha sido restringido debido a la alta mortandad de las vitroplantas al ser transferidas a condiciones *ex vitro*. Los protocolos desarrollados para aumentar el porcentaje de sobrevivencia de las vitroplantas de papaya representan serias dificultades para los investigadores. Se ha descrito consecutivamente que el enraizamiento *ex vitro* y la aclimatación son las principales etapas críticas de propagación en *C. papaya*. Las plantas obtenidas *in vitro* al ser trasplantadas a condiciones *ex vitro* deberán mantener una alta humedad relativa cercana al 80 % (Nhut et al., 2003) y tendrán que ser tratadas con enraizadores comerciales en particular durante los primeros 15 días de trasplante, con estas medidas se pretende que los estomas sean funcionales y que las plantas formen un buen sistema radical, debido a que su nutrición dependerá durante mucho tiempo y en gran parte de la funcionalidad de sus raíces (Estrella-Maldonado et al., 2016).

1.1.10. Inducción de raíces por auxinas

Las plantas son organismos bipolares que en la parte apical genera órganos vegetativos y reproductivos y en la parte basal generan el sistema radicular. Las raíces realizan una variedad de funciones biológicas. Mantienen la planta erguida, pero también son el sitio de absorción de agua y nutrientes, además de constituir importantes órganos de almacenamiento en algunas especies. Las raíces producen reguladores de crecimiento como auxinas y metabolitos secundarios, tales como flavonoides y alcaloides que pueden estar implicados en la defensa contra agentes patógenos o contra microorganismos simbióticos. La formación de las raíces es un pre-requisito a largo plazo para la sobrevivencia de las plantas. La inducción de raíces adventicias se ha atribuido a varias hormonas vegetales (Drew et al., 1979; Wample and Reid, 1979). Las auxinas se han reportado de importancia para el enraizamiento en plantas de *Rumex palustris y Rumex thyrsiflorus* usando diferentes concentraciones de Ácido Indol Butírico (IBA), con esta fitohormona se indujeron la formación de raíces adventicias *in vitro* (Visser et al., 1995).

La formación de raíces laterales puede ser inducida aumentando el contenido de auxina (Jönsson et al., 2006) y más concretamente por la activación local de la síntesis de auxina en las células del periciclo (Sick et al., 2006). En principio, hay dos procesos que tienen el potencial de modificar el nivel de cualquier compuesto en una célula: el transporte polar y su regulación. En efecto, para la auxina, ambos cambios en el transporte de la auxina así como en la regulación se han demostrado estar involucrados en la modulación de desarrollo, en particular en raíces de las plantas (Petrásek and Friml, 2009; Vanneste and Friml, 2009).

La distribución de auxina nativa (típicamente Ácido Indol-3-Acético) en el cuerpo de la planta se realiza a través de distancias cortas y largas (por ejemplo, entre las células adyacentes, así como entre el ápice del brote y los sitios de iniciación de raíces laterales respectivamente (Zazímalová et al., 2010), sin embargo, la clave para entender cómo la auxina puede moverse a través de la membrana plasmática (MP) se encuentra en su naturaleza físico-química. Debido a que todas las auxinas son ácidos débiles, su forma molecular (protones disociados o no disociados) y por lo tanto su capacidad de penetrar a través de la membrana depende del pH. En las plantas, el pH apoplástico es aproximadamente de 5.5 como resultado de protones extruidos por la MP H^+-ATPasa. A este pH, el equilibrio de las moléculas de IAA (pKa = 4.85) se calcula que es aproximadamente 83 % disociado (aniónico, A-) mientras que el 17 % de los protones está asociado (es decir, no disociado). Aunque la naturaleza hidrófoba del grupo indol del IAA permite la asociación de la molécula con la superficie de la MP, la carga negativa de un grupo carboxilo disociado en la molécula evitará que atraviese la membrana. Como tal, sólo el protón-asociado (no disociada) de moléculas IAA pueden entrar en la célula por difusión lipófilico (movimiento pasivo) a través de la MP sin la ayuda de una proteína transportadora (Zazímalová et al., 2010). En el citoplasma de las células vegetales, el pH es de aproximadamente 7; a este pH, el equilibrio de las moléculas de auxina se desplaza de forma aniónica, disociada. Es así como los transportadores de auxina direccionan el flujo celular de la auxina y que acoplados a las células adyacentes puede establecer los flujos polares. Este concepto es la base del modelo de difusión quimiosmótica polar o hipótesis quimiosmótica (Rubery and Sheldrake, 1974; Goldsmith, 1977). Es así como múltiples proteínas de transporte están obligadas a mantener los flujos direccionales de la auxina dentro de órganos y tejidos (Zazímalová et al., 2010).

1.1.11. Auxinas

El nombre auxina significa en griego “crecer” y se le atribuye a un grupo de compuestos que estimulan la elongación de las células. En el siglo XIX, Theophyl Ciesielski demostró que un estímulo transmitido desde el ápice era el responsable del crecimiento de la raíz pero fue Fritz Went en 1926 quien a partir de la avena aisló esta molécula promotora del crecimiento (Kende and Zeevaart, 1997; Azcón and Talón, 2008).

La auxina es una sustancia de bajo peso molecular, derivada de triptófano (Ljung et al., 2002), asimismo son una clase de fitohormonas de plantas o reguladores del crecimiento que coordinan los principales procesos de desarrollo de las plantas incluyendo la embriogénesis, la diferenciación del tejido vascular, el mantenimiento del meristemo y la iniciación de los órganos laterales (raíces laterales, brotes axilares, flores) (Vanneste and Friml, 2009). La auxina más importante presente en la mayoría de las plantas es el Ácido Indol-3-Acético (IAA), esta fitohormona se sintetiza principalmente en los primordios de las hojas, en las hojas jóvenes y en las semillas en desarrollo, además, son transportadas hasta la raíz. El IAA controla la división celular, la elongación y la diferenciación (Worley et al., 2000), las respuestas trópico, dominancia apical, la división celular cambial, iniciación de las raíces y la diferenciación de tejido vascular (Hagen and Guilfoyle., 2002). Existen otros compuestos con actividad auxínica en las plantas, como el Ácido Fenil-Acético (PAA) y 4-Cloro-Indol-Acético (4-CI-AA) pero se conoce poco su papel fisiológico. Existen, además, auxinas sintéticas como el ácido 2.4-Diclorofenoxiacético (2,4-D) utilizado para promover la diferenciación celular, el Ácido Indol Butírico (IBA) y Ácido Naftalen-1-Acético (ANA) que son comúnmente utilizados para inducir el enraizamiento y el desarrollo del fruto (Salisbury et al., 1994).

Las señales de la auxina se transfieren rápidamente en una variedad de respuestas que incluyen cambios en la dirección del crecimiento, formación de brotes, ramificación de las raíces y la diferenciación vascular (Leyser, 2001). La auxina se transporta a través de la planta de forma asimétrica por proteínas de membrana llamados transportadores de entrada y salida de auxina (Estelle, 2001). La auxina es transportada basipetalmente de los brotes a la base de la planta y hacia abajo a través de las raíces. La concentración de

auxina en una célula determina en gran medida los efectos que tiene sobre la célula (Leyser and Berleth, 1999).

El IAA se ha estudiado durante años, pero las vías detrás de la biosíntesis del IAA y la forma en que es detectado por las células no se conoce bien (Bartel, 1997). Una vez producido el IAA, se transporta a través de la planta mediante el transporte polar de auxina (PAT) desde el meristemo apical de las hojas hacia el meristemo radicular. Los mecanismos de detección de auxina por células están todavía bajo investigación. El PAT se cree regula una serie de procesos de desarrollo, incluyendo el tropismo y la dominancia apical. Las plantas regulan concentraciones de IAA mediante la conjugación de IAA para aminoácidos o azúcares, y/o convierten el triptófano (un precursor IAA) a IAA para mantener IAA libre a niveles constantes (Hobbie and Estelle, 1994).

1.1.12. Biosíntesis y señalización de las auxinas

Experimentos genéticos y bioquímicos han demostrado la existencia de dos rutas: una ruta dependiente de triptófano y otra independiente de triptófano (Figura. 1.1). De igual forma, se han propuesto rutas dependientes de triptófano denominadas según el intermediario: la ruta del Ácido Indol-Pirúvico (IPA), la ruta del Indol-3-Acetamida (IAM), la ruta de la triptamina, y la ruta del Indol-3-Acetaldoxima (IAOx). La ruta dependiente de triptófano es la del ácido indol-pirúvico (ruta IPA), en la que se produce la transaminación del triptófano para producir Ácido-3-Pirúvico (IPA) por la acción de una triptófano aminotransferasa. A continuación, IPA se descarboxila por medio de la enzima ácido Indol-3-Pirúvico descarboxilasa para dar lugar al Indol-3-Acetaldehído (IAAld); la oxidación del aldehído a ácido produce IAA en una reacción catalizada por la Indol-3-Acetaldehído oxidasa (Woodward and Bartel, 2005). Recientemente, la caracterización de mutantes de *A. thaliana* para el estudio de la interacción etileno-auxinas a nivel tisular y de los mutantes *sav3* llamados mutantes del SAS (“shade avoidance syndrome”), fueron incapaces de elongar sus tallos en condiciones de luz de baja intensidad, lo cual han permitido la identificación de la enzima Triptófano-Aminotransferasa1 (TAA1), que al parecer está implicada en formar Ácido-3-Pirúvico (IPA) a partir de triptófano (Stepanova et al., 2008; Tao et al., 2008). La triptamina producida por descarboxilación del triptófano puede dar lugar también al IAA a través de la formación del intermediario Indol-3-

Acetaldoxima (IAOx). La identificación de *yucca*, un mutante que acumula IAA sugiere que esta ruta de la triptamina puede darse en varias especies de plantas. El fenotipo de *yucca* deriva de la sobreexpresión de una enzima parecida a la Flavin-Monoxigenasa (FMO) que oxida triptamina a N-Hidroxinil-Triptamina (NHT) *in vitro* (Woodward and Bartel, 2005). Igualmente, Indol-3-Acetaldoxima (IAOx) puede derivar de la descarboxilación oxidativa del triptófano y puede dar lugar a IAA a través de dos intermediarios principales: el Indol-3-Acetaldehído (IAAld) y el Indol-3-Acetonitrilo (IAN) que es convertido a IAA por la enzima nitrilasa. El triptófano también puede dar lugar a IAA a través de la ruta de la Indol-3-Acetamida (IAM) mediante la acción enzimática de una IAM hidrolasa. El hecho de que mutantes deficientes en triptófano acumularan IAA (mutantes *trp2* y *trp3* en *A. thaliana*) sugirió una ruta alternativa triptófano-independiente en la que el IAA sería sintetizado a partir de indol o indol-glicerol fosfato (Woodward and Bartel, 2005).

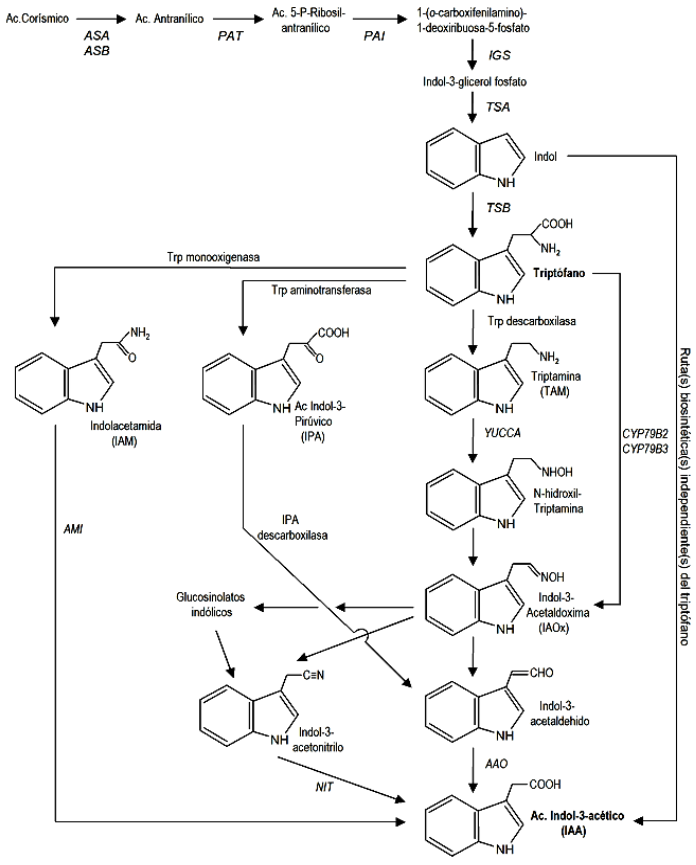


Figura 1.1. Rutas de biosíntesis del IAA. Las enzimas aparecen con el nombre de la supuesta enzima que catalizaría la reacción o con el nombre del gen representativo identificado en *Arabidopsis* (Jordán and Casaretto, 2006).

1.1.13. Distribución y regulación de las auxinas

La distribución de la auxina en las plantas se produce a través de dos tipos de transportes distintos: el primero es un transporte no polar (rápido) a través del floema, el segundo es un transporte polar (PAT, Transporte Polar de Auxina), el cual es lento transportándose de célula en célula en varios tejidos. El movimiento del IAA mediante PAT está mediada por proteínas de transporte situados en la MP. Estas proteínas de transporte del IAA incluyen transportadores de entrada (AUX1/LAX; AUXIN RESISTENTE 1) (Bennett et al., 1996; Swarup et al., 2008) y transportadores de salida (PIN; PINFORMED) (Galweiler et al., 1998; Noh et al., 2001; Teale et al., 2006; Zazimalova et al., 2010). La localización asimétrica de las proteínas AUX1/LAX y PIN desempeñan un papel crítico en la polaridad del transporte de IAA y su función es importante para mantener un adecuado transporte polar de auxina (IAA) a través de la MP (Muday and DeLong, 2001), por lo anterior, un adecuado PAT es necesario para la formación y el mantenimiento de niveles locales de de auxina que involucra los procesos organogénicos, desarrollo de patrones y respuestas diferenciales de crecimiento en las plantas (Tanaka et al., 2006). En la Figura 1.2 (A), se representa la distribución de la auxina mediante su transporte polar, se observan moléculas de IAA que al no estar disociadas entran a las células por difusión pasiva (a), mientras que los aniones, auxinas disociadas menos permeables (IAA) se transportan por transportadores de la familia AUX1/LAX (b). En el medio intracelular (c), IAA se disocia y requiere del transporte activo a través de transportadores de salida PIN para salir de la célula (Mravec et al., 2009). La actividad de transportadores PIN utiliza un gradiente H^+ por acción de la MP en H^+-ATPasa (d), posiblemente también la pirofosfatasa vacuolar de los transportadores ABCB tienen actividad ATPasa (e) (Li et al., 2005).

Por otra parte, durante el mecanismo de regulación molecular, las auxinas regulan la expresión de genes diana mediante la degradación de las proteínas nucleares de vida corta conocidos como Aux/IAA. Las auxinas promueven la ubiquitinación de estas proteínas por el complejo SCF^{TIR1} (Skp1-Cullin-Fbox) que conduce a su degradación por el proteasoma 26S, de esta manera se elimina el efecto represor de los complejos Aux/IAA sobre los factores de transcripción de respuesta a auxinas conocidos como ARFs y se permite la transcripción de genes diana de las auxinas. El mecanismo por el cual las auxinas promueven la interacción SCF^{TIR1}-Aux/IAA es la unión directa de IAA a TIR1, que

es el receptor de auxinas (Dharmasiri et al., 2005a; Kepinsky and Leyser, 2005; Bishopp et al., 2006). En la Figura 1.2 (B), la auxina intracelular se une a su receptor nuclear de respuesta de las proteínas F-box, (TRANSPORT INHIBITOR RESPONSE 1/AUX SIGNALING F-BOX; TIR1/AFB) que son subunidades de los complejos proteína SCF E3-ligasa (a), esto conduce a la ubiquitinación (Ub) y degradación mediada por el proteasoma de los represores transcripcionales Aux/IAA (b), posteriormente los ARFS son desreprimidos y activan la expresión génica inducible por auxina (c) (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005), asimismo los transportadores de auxina están regulados por este mecanismo (d).

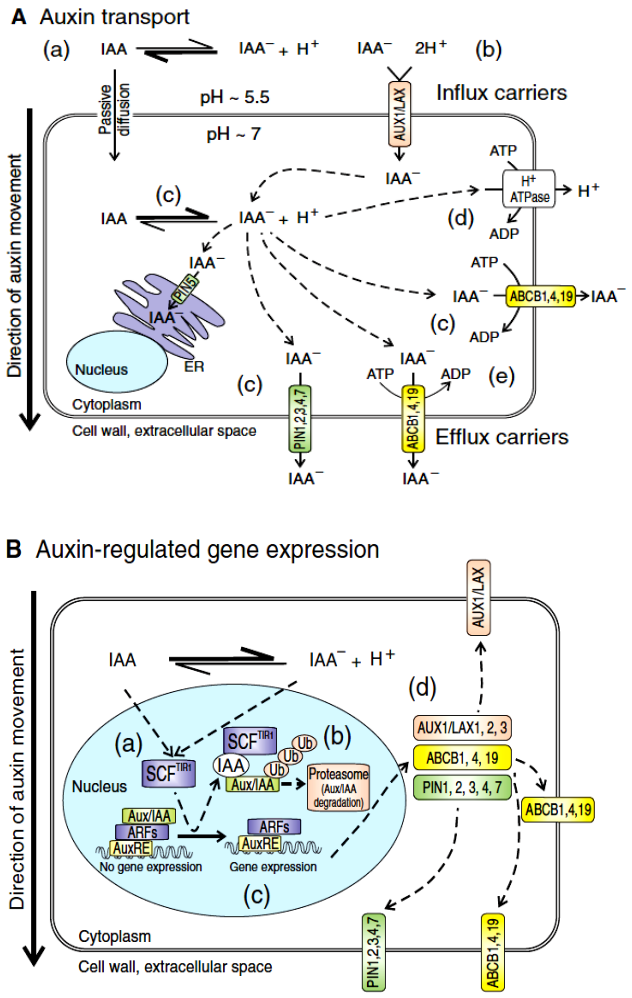


Figura 1.2. Representación esquemática del transporte de auxina a través de la membrana plasmática **(A)**. Representación esquemática de la expresión génica regulada por auxina (**B**) (Petrásek and Friml, 2009).

1.1.14. Transportadores de auxina

Como se ha mencionado anteriormente, las moléculas de auxina pueden entrar en las células pasivamente, sin embargo, también pueden ser transportadas dentro de las células a través de la actividad de H^+ gracias a familias transportadoras de flujo de entrada **AUX/LAXs** y de flujo de salida **PINs** (Kerr and Bennett, 2007), ambas familias tienen una función directa sobre la formación de la polaridad celular, por lo tanto, la comprensión de los mecanismos de su ubicación polar es importante (Medvedev, 2012). Ambas familias son las responsables del transporte de auxina tanto en los brotes de la planta como en las raíces. Es así como la sobre-expresión de AUX/LAXs mejora la distribución de la auxina en las raíces laterales, mientras que la baja-regulación de PINs modula la separación de las raíces a lo largo del eje de la raíz principal (Grieneisen et al., 2007).

1.1.14.1. AUX1/LAXs

En el genoma de *A. thaliana* se ha identificado un AUX1 y tres homólogos a proteínas LAX (LAX1, LAX2, y LAX3) (Parry et al., 2001). Miembros de esta familia comparten una similitud de secuencia de aminoácidos de aproximadamente el 80 % (Parry et al., 2001). Los transportadores AUX/LAXs son necesarios para la captación activa de la auxina en procesos celulares de alta y rápida afluencia de esta fitohormona (Kramer and Bennett, 2006), sin embargo es posible que algunos de los transportadores descritos anteriormente pueden ser multifuncionales y que el número de transportadores implicados en estos procesos pudiera variar entre las diferentes especies (Zazímalová et al., 2010).

1.1.14.2. PINs

La polarización de proteínas PIN en la membrana celular y su actividad determinan la dirección del flujo de la auxina y proporcionan su formación local para el inicio del órgano y, finalmente de la morfogénesis (Blakeslee et al., 2005). Por tal motivo, las proteínas PIN desempeñan un papel importante en la regulación de auxina en las plantas (Habets and Offringa, 2014). En *A. thaliana*, miembros PINs fueron detectados a través del análisis de las anomalías fenotípicas mediante la pérdida de función (mutantes) y hasta la fecha esta

familia se compone de ocho miembros y se divide en dos subclados básicos que difieren en la longitud de un bucle hidrófilo en el medio de su cadena polipéptidica. Los PINs "de distancia larga" tienen su bucle central hidrófilo ya sea parcial o significativamente alargado (PIN1, 2, 3 ,4 y 7) (Tanaka et al., 2006; Vieten et al., 2007; Zazímalová et al., 2007) indicando genes que en su mayoría muestran localización polar en la MP la cual determina la dirección del flujo polar de auxina (Petrásek *et al.*, 2006; Wisniewska et al., 2006; Yang and Murphy, 2009). En contraste con los transportadores de distancia larga, hay tres miembros de la familia PINs de “distancia corta” que tienen su bucle central hidrófilo ya sea parcial o significativamente reducido (PIN5, 6 y 8). La subfamilia de PINs de "distancia corta" se caracteriza por la ausencia del bucle hidrofílico (Krecek et al., 2009), se localizan en el retículo endoplásmico (RE) (Mravec et al., 2009) y proporcionan una distribución intracelular de auxina entre el citoplasma y el lumen (PIN5, PIN6), entre el citoplasma y las vacuolas (PIN8), así como la homeostasis de auxina en las células (Mravec et al., 2009). Ninguna de las secuencias de los PINs de “distancia corta” contienen un dominio de unión al ATP y por esta razón son considerados como transportadores secundarios. De manera general, los representantes PINs solo se han observado en genomas de las plantas superiores (Mravec et al., 2009), sin embargo la secuencia de aminoácidos de miembros de la familia PIN presentan una tipología trans-membranal similar a algunas proteínas transportadoras (Yang and Murphy, 2009).

1.1.15. Factores de transcripción de auxina

Los factores de transcripción (FTs) son proteínas que se unen a secuencias específicas en el ADN cerca de sus genes diana, modulando así la iniciación de la transcripción. FTs pueden activar o reprimir la transcripción dependiendo de donde se unen en relación al sitio de inicio de la transcripción del gen diana (Browning and Busby, 2004). Cada FT regula un conjunto de genes en respuesta a las condiciones ambientales y/o activadores intracelulares. Una interacción reguladora de la transcripción entre un FT y su gen diana comprende: (1) la señal de detección, (2) la transducción de señales, (3) el FT y (4) su gen (s) diana (Salgado et al., 2007).

Hay muchos FTs que intervienen en el control a gran escala como la floración, ramificación, enraizamiento y formación de la madera. Los genes que se activan o reprimen durante este proceso y son los responsables de los muchos efectos fisiológicos de la auxina.

En este sentido, la auxina es percibida por dos tipos de familias de proteínas que funcionan como receptores: la primera familia se localizan en la membrana plasmática como en el retículo endoplásmico (ABP1; AUXIN BINDING PROTEIN 1), la segunda familia se localizan en el núcleo (TIR1/auxin-signaling F box proteins (AFBs) (Finet and Jaillais, 2012; Ljung, 2013; Sauer et al., 2013). Dentro de la familia de los ABP, ABP1 es una proteína esencial ya que su mutación es letal en la etapa embrionaria (Tanaka et al., 2006); este transportador es importante durante la división y elongación celular, y durante el desarrollo embrionario (Ljung, 2013; Sauer et al., 2013). Una vez que las auxinas entran en la célula, desencadenan una serie de procesos que terminan en la regulación de la expresión de genes diana de respuesta a auxinas (Vanneste and Friml, 2009; Sauer et al., 2013). Esta vía de señalización involucra a dos familias de proteínas: las Auxin/Indole-3-Acetic Acid (Aux/IAA) y las Auxin Response Factor (ARF). Estas dos familias de genes han sido bien estudiados en *A. thaliana,* se han encontrado que desempeñan un papel en el control de la regulación de genes inducidos por auxina, las proteínas Aux/IAA constan de 29 miembros y las proteinas ARFs constan de 23 miembros (Remington et al., 2004; Overvoorde et al., 2005).

Miembros de estas dos familias forman dímeros con una alta afinidad entre ellos (Tanaka et al., 2006). Lla auxina es la responsable de regular estas interacciones proteicas de dos maneras, por un lado, regula de manera positiva la transcripción de los genes que codifican para proteínas represoras IAA, por otro lado, promueve la degradación de represores Aux/IAA mediante la ubiquitinación y proteólisis de los mismos (Tanaka et al., 2006; Finet and Jaillais, 2012; Ljung, 2013). El mecanismo propuesto para la degradación de las Aux/IAA es el siguiente: en condiciones de baja concentración de auxina, las proteínas represoras Aux/IAA forman dímeros con las proteínas ARFs e impiden su acción como factores transcripcionales (Tanaka et al., 2006; Finet and Jaillais, 2012; Ljung, 2013). Una vez que se incrementa la concentración de IAA (Kd ~20-80 nM), la auxina se une a alguno de los cuatro receptores nucleares (TIR1/AFB) que son proteínas

con caja F y que junto con otras proteínas (ASK1, CUL1 y RBX) forman un complejo de ubiquitinación ($SCF^{TIR1/AFB}$) que reconoce y degrada a represores Aux/IAA mediante la vía del proteosoma 26S (Tanaka et al., 2006). Una vez que los ARFs están libres por no estar unidos a sus represores Aux/IAA, forman homodímeros estables que activan o reprimen la expresión de los genes diana uniéndose a la caja Auxin Response Element (ARE) de los mismos (Tanaka et al., 2006; Finet and Jaillais, 2012; Ljung, 2013).

Las proteínas de estas dos familias tienen una estructura de cuatro dominios. En el caso de las Aux/IAA, en el primer dominio del extremo amino terminal tiene un motivo denominado EAR (Tanaka et al., 2006) que es esencial para su papel represor. El dominio II es esencial para la degradación de estas proteínas mediada por las auxinas y los últimos dos dominios (III y IV) son esenciales para las interacciones proteína-proteína, tanto con los ARFs como con otras proteínas que funcionan como reguladores transcripcionales (Finet and Jaillais, 2012; Ljung, 2013).

Los ARFs poseen un dominio de unión a DNA pero algunos de ellos (ARF3, ARF13 y ARF17) carecen del dominio carboxilo terminal que es importante para las interacciones proteína-proteína, por lo tanto, no están regulados por la cantidad de auxina presente en la célula (Paponov et al., 2008). Los ARFs pueden ser tanto activadores como represores de la transcripción; si presentan una región a la mitad de la proteína que sea rica en Glu/Ser/Leu (QSL) actúan como activadores, mientras que si presentan un dominio rico en Ser/Pro/Gli actúan como represores (Tanaka et al., 2006). Al igual que las proteínas Aux/IAA, los dominios III y IV son los importantes para que se lleven a cabo las interacciones protéicas y son los responsables de las interacciones dentro y entre familias (Tanaka et al., 2006; Finet and Jaillais, 2012; Ljung, 2013).

1.1.15.1. Aux/IAA

La familia Aux/IAA es un grupo genes de respuesta temprana a la auxina. En particular, la familia de genes Aux/IAA ha sido ampliamente estudiada, incluyendo su regulación y su transcripción. Genes Aux/IAA son exclusivos del reino vegetal, sin embargo, se conservan entre monocotiledóneas, dicotiledóneas y gimnospermas (Hagen and Guilfoyle, 2002).

Los genes Aux/IAA fueron aislados por primera vez como ARNm de respuesta a auxina a partir de la soja (Walker and Key, 1982). Esta familia en *A. thaliana* son inducidos en diferentes momentos después de la adición de la auxina, es así como AtAux/IAA1, AtAux/IAA2, AtAux/IAA3, AtAux/IAA5 y AtAux/IAA6 son inducidos cinco minutos después de la adición de auxina exógena, mientras que AtAux/IAA7, AtAux/IAA8, AtAux/IAA9 y AtAux/IAA10 son inducidos treinta minutos después de la adición de auxinas (Park et al., 2002). No es probable que sean las diferencias en las células y/o patrones de expresión específico de tejido para diferentes genes ARFs y Aux/IAA (Ulmasov et al*.,* 1999a). Esto reduce el número de ARFs y Aux/IAA que se expresan al mismo tiempo, reduciendo de este modo el número de interacciones que pueden ocurrir en la planta. Aux/IAA están específicamente inducidas por la auxina en la célula (Hagen and Guilfoyle, 2002).

Los genes Aux/IAA tienen cuatro dominios conservados numerados del uno al cuatro. Los dominios del I al IV tienen 8, 14, 17, y 40 residuos respectivamente (Guilfoyle et al., 1998b). La función del dominio I sigue siendo desconocido, pero el dominio II puede interactuar con el fitocromo A y está involucrado en la regulación de la fotosíntesis (Liscum and Reed, 2002). El dominio I también podría desempeñar un papel en la homodimerización de proteínas Aux/IAA (Ouellet et al., 2001). El dominio II es responsable de la desestabilización de la proteína, probablemente a través de interacciones con la vía de la ubiquitina (Worley et al., 2000; Colon-Carmona et al., 2000). La degradación de la vía ubiquitina proteasoma se cree que es el principal regulador de la carencia y/o abundancia de proteínas Aux/IAA en la célula. Si las proteínas Aux/IAA son regulados por esta vía, se puede explicar en parte por qué las proteínas Aux/IAA son de corta duración en el interior de la célula. El dominio II se conserva de 24 a 29 genes Aux/IAA en *A. thaliana*, por lo que es razonable plantear la hipótesis de que las proteínas Aux/IAA son degradados por esta vía (Gray et al., 2001). La rápida degradación de las proteínas Aux/IAA es necesario para la respuesta normal a la auxina (Worley et al., 2000). Nueve diferentes mutaciones conocidas en los genes Aux/IAA involucran una sola sustitución de un aminoácido en el dominio II y funcionan para estabilizar a la proteína (Liscum and Reed, 2002). Estas mutaciones producen severos desarrollos en los fenotipos, destacando el importante papel que tiene Aux/IAA en el desarrollo de la planta (Liscum and Reed, 2002). Los genes Aux/IAA del híbrido álamo (*Populus tremula* L. x *Populus tremuloides michx.*) PtAux/IAA3 y PtAux/IAA4 tienen una sustitución de una

glicina por un ácido aspártico en el dominio II, que se sabe que regulan la estabilidad de las proteínas de IAA (Moyle et al., 2002). El dominio II supuestamente tiene una función adicional como una señal de localización nuclear. Parte de dominio II y los residuos conservados KR (Lisina y Arginina) entre los dominios I y II en casi todos los genes Aux/IAA se cree que funcionan como señales de localización nuclear (Moyle et al., 2002). El dominio III y IV son dominios putativos de interacción proteína-proteína. Estos dominios son reportados para permitir la interacción entre proteínas Aux/IAA con proteínas ARFs (Figura I.3). El dominio III se ha reportado que tiene otras funciones más allá de la formación de dímeros proteína-proteína. Se predice que tienen un pliegue anfipático βαα similar al pliegue βαα de los represores transcripcionales en procariotas, tales como Arco y Metj (Abel et al., 1994). En procariotas, este pliegue βαα modula la dimerización de los monómeros de Arc y el reconocimiento ADN del operador en los sitios medios por dímeros de Arc. Es posible que las proteínas Aux/IAA también puedan regular la transcripción de la expresión secundaria génica en respuesta a auxina (Morgan et al., 1999).

1.1.15.2. ARFs

Los factores de respuesta a auxina (ARFs) son factores de transcripción que regulan la expresión de genes en respuesta a la auxina (Guilfoyle and Hagen 2001; Tiwari et al., 2003). El movimiento de la auxina dentro y fuera de las células por el transporte polar de auxina es el control primario de la concentración de auxina en las células. Las proteínas ARFs han demostrado su interacción con otras proteínas para regular la transcripción de la familia de genes Aux/IAA y de otras familias de genes (Guilfoyle et al., 1998a; Hagen and Guilfoyle, 2002). Los genes Aux/IAA son inducidos por la auxina (Abel et al., 1995), mientras que la familia de proteínas ARFs no se ha encontrado que sufran cambios transcripcionales dependientes de la auxina (Ulmasov et al., 1999b).

Hay 23 genes ARFs en el genoma de *Arabidopsis* que varían en tamaño de 64 a 129 kD (Guilfoyle et al., 1998b; Hagen and Guilfoyle, 2002). AtARF23 es más probable un pseudogen, debido a la presencia de un codón de paro en el dominio de unión al DNA (DBD) y una falta de secuencia corriente abajo del DBD. Además, los AtARFs 12, 13, 14, 15, 20, 21 y 22 pueden ser pseudogenes porque no hay EST o mRNA reportados para

estos genes (Guilfoyle et al., 1998b). En estos genes todo el mapa posee una pequeña región en el cromosoma 1 y tienen secuencias de ADN estrechamente relacionados (Hagen and Guilfoyle, 2002). AtARF3 carece de los dominios III y IV carboxilo-terminal que se utiliza para la homo y heterodimerización de los ARF con las proteínas Aux/IAA (Liscum and Reed, 2002). Se cree que AtARF3 no es capaz de formar dímeros con otros ARFs o con las proteínas Aux/IAA. Si AtARF3 es capaz de interactuar con otras proteínas es más probable que sea a través de una proteína de acoplamiento desconocido y/o de algún otro factor (Liscum and Reed, 2002). Los restantes ARFs se creen ser biológicamente activos.

Las proteínas ARFs contienen un dominio de unión al ADN (DBD), una región media (MR) que separa el DBD, del dominio III y dominio IV (Figura 1.3). El DBD se ha demostrado que interactúa con el sitio TGTCTC en AuxRE, un promotor de motivo conservado en los genes inducidos por la auxina (Guilfoyle and Hagen, 2001). La región media se cree que funciona como un activador o represor de la transcripción (Ulmasov et al., 1999a). Los dominios III y IV están implicados en las interacciones con otras proteínas ARFs y Aux/IAA. Los elementos de respuesta a auxina (AuxRE) están corriente arriba del sitio de inicio de muchos genes inducidos por respuesta temprana a la auxina, incluyendo la familia Aux/IAA. El sitio AuxRE puede ser sencilla, sólo pude contener un elemento TGTCTC, o puede tener elementos constitutivos o de acoplamiento, que pudieran afectar aún más la unión de los ARFs (Ulmasov et al., 1999b). Los elementos constitutivos o de acoplamiento presentes pueden influir con el factor de transcripción que se une al elemento TGTCTC (Ulmasov et al., 1999b). Hay dos secuencias conservadas TGTCTC y (G/T) GTCCCAT que se sabe que son los promotores de la respuesta temprana a la auxina, incluidos genes Aux/IAA (Guilfoyle et al., 1998b). En el motivo TGTCTC, los primeros 4 bases son importantes para la unión i*n vitro* de ARF1 y ARF5, mientras que las sustituciones de nucleótidos en la posición 5 y 6 pueden ser tolerados, especialmente en la posición 5, aunque pueden reducir la unión (Guilfoyle et al., 1998b). Los dominios de unión al ADN de proteínas ARFs han sido demostrado que reconocen y se unen específicamente a AuxRE (Ulmasov et al., 1999a). El DBD es de aproximadamente 350 aminoácidos de longitud y está altamente conservada a lo largo de la familia de genes ARF, además de ser necesario y para la unión a la AuxRE (Ulmasov et al., 1999b). La región media (MR), entre el DBD y el dominio III, es variable entre los miembros de la

familia ARFs. Ulmasov et al. (1999a) señala que, aunque la MR es variable, existe una preferencia de aminoácidos entre las proteínas ARFs. AtARF5, 6, 7, y 8 tienen una preferencia por los aminoácidos glutamina, leucina y serina (Q /L /S) y son activadores putativos de la transcripción, mientras que AtARF1 tiene una preferencia por prolina, serina y treonina (P /S /T) y es un represor putativo de la transcripción (Ulmasov et al., 1999a). Otros ARFs no parecen tener una preferencia de aminoácidos y no presentan una mayor activación o represión al compararlos con los controles. Los dominios III y IV están involucrados en la formación de dímeros con otras proteínas ARFs y Aux/IAA, que también han conservado sus dominios III y IV. Estos dominios han demostrado interactuar en dos ensayos de hibridización formando tanto homodímeros como heterodímeros (Liscum and Reed, 2002). Los homodímeros ARF-ARF, así como heterodímeros ARF-Aux/IAA son capaces de unirse al ADN de otro ARFs de manera estable dependiendo si la proteína específica ARFs es un represor o activador de la transcripción (Liscum and Reed, 2002).

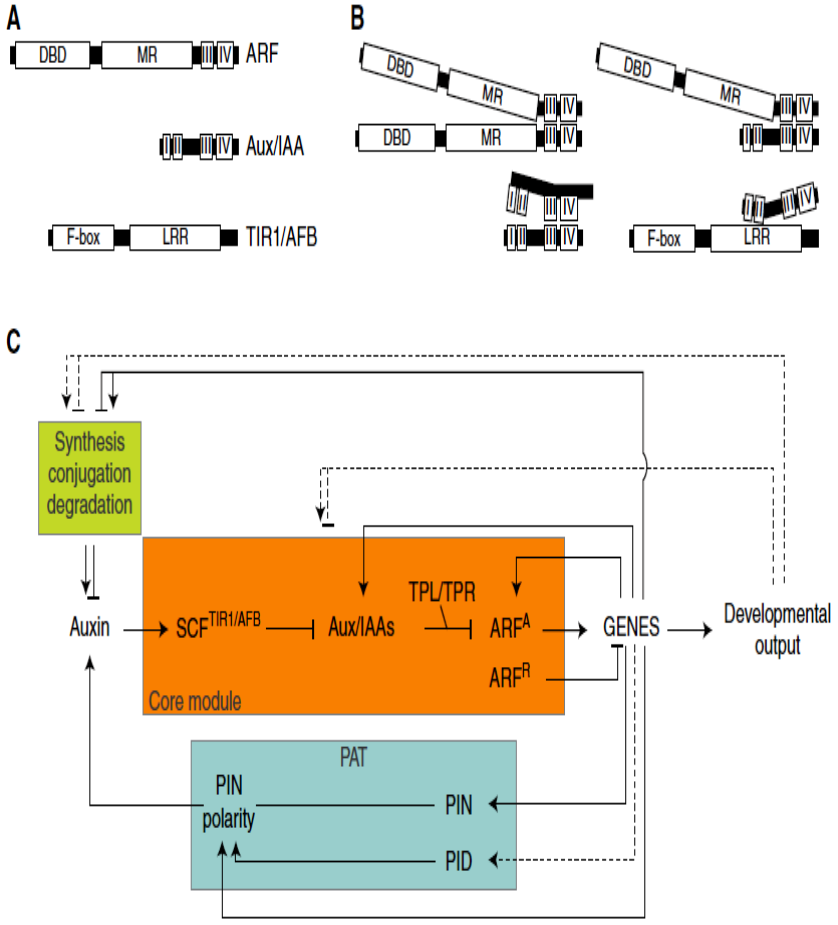


Figura 1.3. Representación esquemática del sistema AFB-Aux/IAA-ARF y sus relaciones de retroalimentación con el transporte polar de auxina (PAT) y el metabolismo de la auxina **(A)** ARF, Aux/IAA, y el dominio de organización TIR1/AFB. **(B)** ARFs y Aux/IAAs pueden interactuar dentro de sus familias a través del dominio III y IV para formar homo- y heterodímeros (izquierda) y la interacción también pueden ocurrir si se involucran a las familias ARFs y Aux/IAAs (parte superior derecha) y Aux/IAAs and TIR1/AFBs (parte de abajo derecha). **(C)** La complejidad de la regulación en respuesta de la auxina. Las líneas que terminan en barras indican inhibición, mientras que las líneas que terminan en puntas de flecha indican la activación. Los posibles mecanismos de acción se indican por líneas discontinuas (Del Bianco and Kepinski, 2011). DBD: dominio de unión al DNA; MR: región media; LRR: región rica en leucina; ARF: proteínas tipo factor de respuesta a auxina; Aux/IAAs: proteínas tipo Auxin/Indole-3-Acetic Acid PIN: proteínas de polarización del transporte auxínico; PID: proteínas PINOID; $SCF^{TIR1/AFB}$: complejo E3 ubiquitina-ligase; TPL: proteína TOPLESS involucrada en polaridad basal-aplical mediada por auxina; TPR: co-represor de proteína TOPLESS.

1.1.16. AUX1/LAXs y PINs en las raíces

El transporte de auxina también es requerido para la iniciación y la elongación de las raíces laterales, sin embargo, los métodos físicos y genéticos pueden bloquear el flujo de auxina (Reed et al., 1998; Casimiro et al., 2001). Los defectos en AUX1/LAXs y PINs reducen la iniciación y/o elongación de las raíces laterales debidas a la reducción del movimiento de la auxina (Marchant et al., 2002; Benkova et al., 2003; Wu et al., 2007; Swarup et al., 2008). El desarrollo de las raíces laterales se ha demostrado que requieren cambios complejos en la expresión de las proteínas de tipo PINs en el desarrollo de primordios (Benkova et al., 2003; Sauer et al., 2006).

En un artículo publicado por Laskowski et al. (2008) sugieren que los transportadores de auxina de tipo AUX1/LAXs combinado con la geometría propia de las células, es el mecanismo crucial para la iniciación de las raíces laterales. Asimismo, se ha descrito que altas concentraciones de la auxina en la punta de las raíces en *A. thaliana* es el resultado de un sistema de reflujo impulsado por el transporte de la auxina influenciado por varios miembros transportadores de la familia de proteínas PINs (Dubrovsky et al., 2008).

En experimentos previos realizados en plantas de *A. thaliana* se ha elucidado que AUX1 and LAX3 juegan un papel importante en la redirección de las corrientes polares de la auxina en la tapa lateral de la raíz (Kramer and Bennett, 2006). Por otra parte, PIN2 parece estar estrechamente involucrado en el transporte de la auxina en las zonas de elongación donde se acumula auxina provocando la inducción de la elongación celular y el crecimiento de las raíces en general (Růžička et al., 2007). Además una alta expresión de PIN3 y PIN7 con la proteína GFP son necesarias para iniciar las raíces laterales, mientras que miembros AUX1/LAXs con YFP incrementan la formación de las raíces, es así como un alto gradiente de auxina impulsa la formación de primordios. Estos resultados sugieren que la regulación de ambas familias de proteínas transportadoras modulan el transporte de la auxina la cual es importante en el desarrollo de las raíces laterales (Laskowski et al., 2008).

1.1.17. Aux/IAA y ARFs en las raíces

En *A. thaliana* la organización de la raíz primaria es simple con un número muy conservado de células en cada capa, cualquier cambio puede ser fácilmente detectado. El estudio de mutantes que afectan a este patrón de desarrollo ha permitido el descubrimiento de genes implicados en el desarrollo de la raíz. Algunos de estos genes codifican a factores de transcripción (FTs) que interactúan con elementos cis-acting de ADN u otros reguladores de transcripción para activar o reprimir la expresión de genes diana. Estos FTs regulan cascadas de genes que pueden conducir a importantes cambios morfológicos, fisiológicos y/o metabólicos. FTs actuales en plantas se clasifican de acuerdo con su dominio de unión al ADN, se encuentran : ARFs (factor de respuesta auxina) y proteínas Aux/IAA (Auxina/Ácido Indol-3-Acetico) (Liu et al., 1999), estos FTs regulan la transcripción de la auxina dando la oportunidad de descifrar genes reguladores que controlan los programas de desarrollo que se consideran importantes para comprender la diferenciación del tejido radicular y su desarrollo en respuesta a reguladores de crecimiento (Figura 1.4) (Montiel et al., 2004).

Teniendo en cuenta el mínimo cambio fenotipo de las raíces laterales de varios mutantes Aux/IAA, tales como *iaa14/solitary root (slr)* (Fukaki et al., 2002), *iaa3/short* del hipocotilo o supresor de *hy2 (shy2)* (Tian and Reed, 1999), *iaa19/massugu2 (msg2)* (Tatematsu et al., 2004), y *iaa28* (Rogg et al., 2001), es evidente que proteínas Aux/IAA inhiben la actividad de ARFs requeridos para la formación de raíces laterales. Enfoques genéticos han identificado a AtAux/ARF7 y At Aux/ARF19 como componentes clave de una vía de desarrollo para la regulación de la formación de las raíces laterales. Dobles mutantes *arf7-arf19* exhiben un fenotipo de formación de raíces laterales severamente reducida no observado en mutantes individuales *arf7* y *arf19*, lo que indica que la formación de raíces laterales se regula por estos dos activadores transcripcionales (Weijers et al., 2005; Wilmoth et al., 2005; Li et al., 2006). Por otra parte, ambos ARF7 y ARF19 interactúan con IAA14/SLR, IAA3/SHY2, IAA19/MSG2 y IAA28 en la levadura (Tatematsu et al., 2004; Fukaki et al., 2005; Weijers et al., 2005) y sus patrones de expresión son altamente sobre-expresados (Fukaki et al., 2002; Okushima et al., 2005). Por lo tanto, ARF7 y ARF19 controlan la formación de raíces laterales (Figura 1.4) junto con varios represores transcripcionales Aux/IAA, como IAA14/SLR, IAA3/SHY2, IAA19/MSG2 y IAA28.

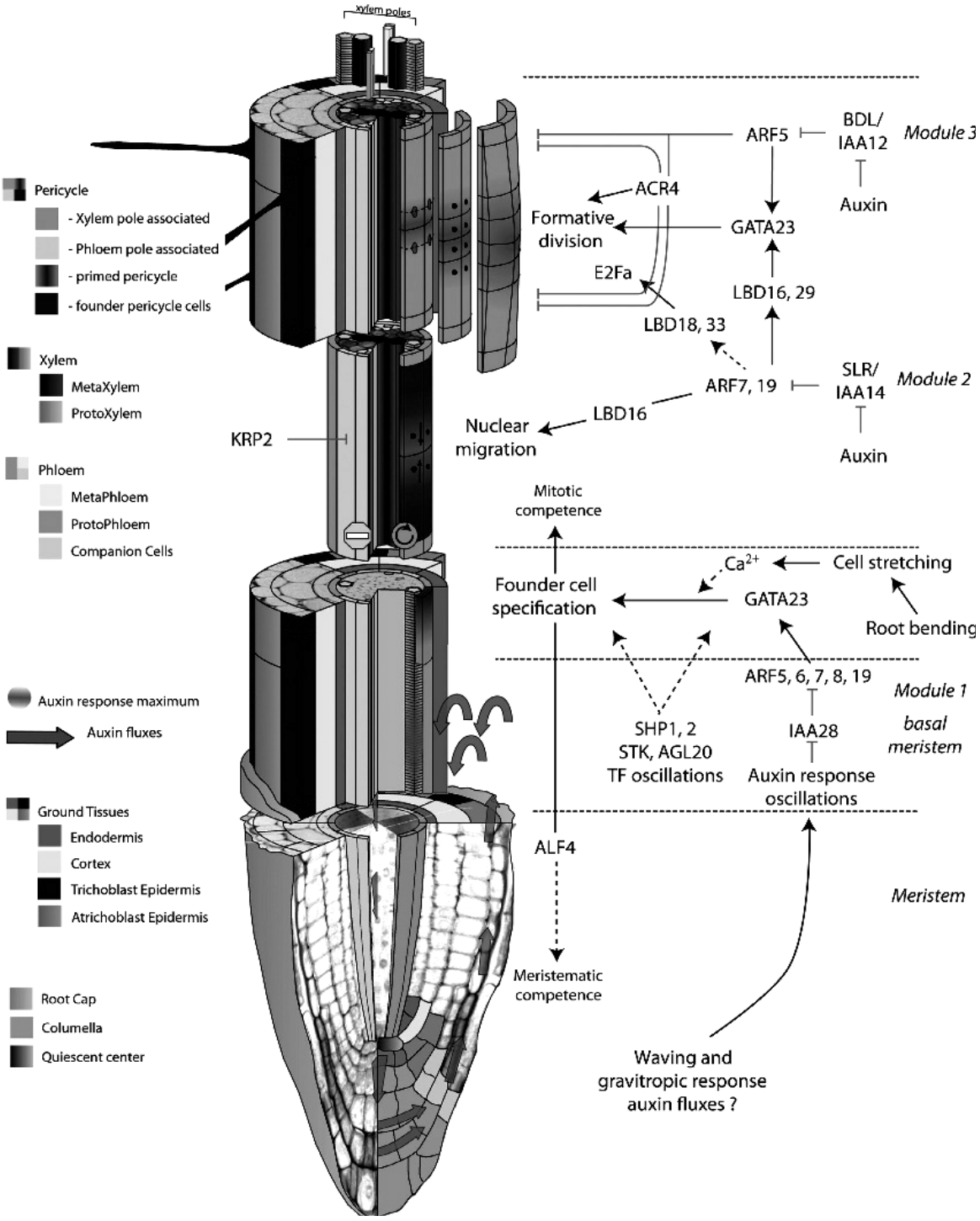


Figura 1.4. Estructura de la raíz primaria y sus diferentes capas para la iniciación de raíces laterales. Se observa los diferentes FTs que intervienen en este proceso (Parizot and Beeckman, 2012).

1.2. HIPÓTESIS

Los genes reguladores de auxinas (Aux/IAA y ARFs) y los genes involucrados en el transporte polar de auxinas (AUX1/LAXs y PINs) en *Arabidopsis thaliana* presentan patrones de expresión en las zonas meristemáticas de la raíz; por lo tanto, es probable que genes ortólogos CpAux/IAAs, CpARFs, CpAUX1/LAXs y CpPINs en *Carica papaya* L. también presenten expresiones diferenciales en las zonas radiculares en *in vitro* plántulas de *C. papaya* cv. Maradol. Así mismo, se observarán cambios en la expresión de genes involucrados en la regulación y el transporte polar de auxinas en *in vitro* plántulas de *Carica papaya* L. expuestas a diferentes tratamientos rizogénicos.

1.3. OBJETIVO GENERAL

El interés final de este trabajo radica en comprender algunos de los mecanismos moleculares de la rizogénesis, mediante el estudio de los posibles cambios de expresión de genes reguladores de la transcripción de auxinas (CpAux/IAA y CpARFs) y de genes involucrados en el transporte polar de auxinas (CpAUX1/LAXs y CpPINs) en *in vitro* plántulas de papaya (*Carica papaya* L.) cv. Maradol expuestas a diferentes tratamientos rizogénicos.

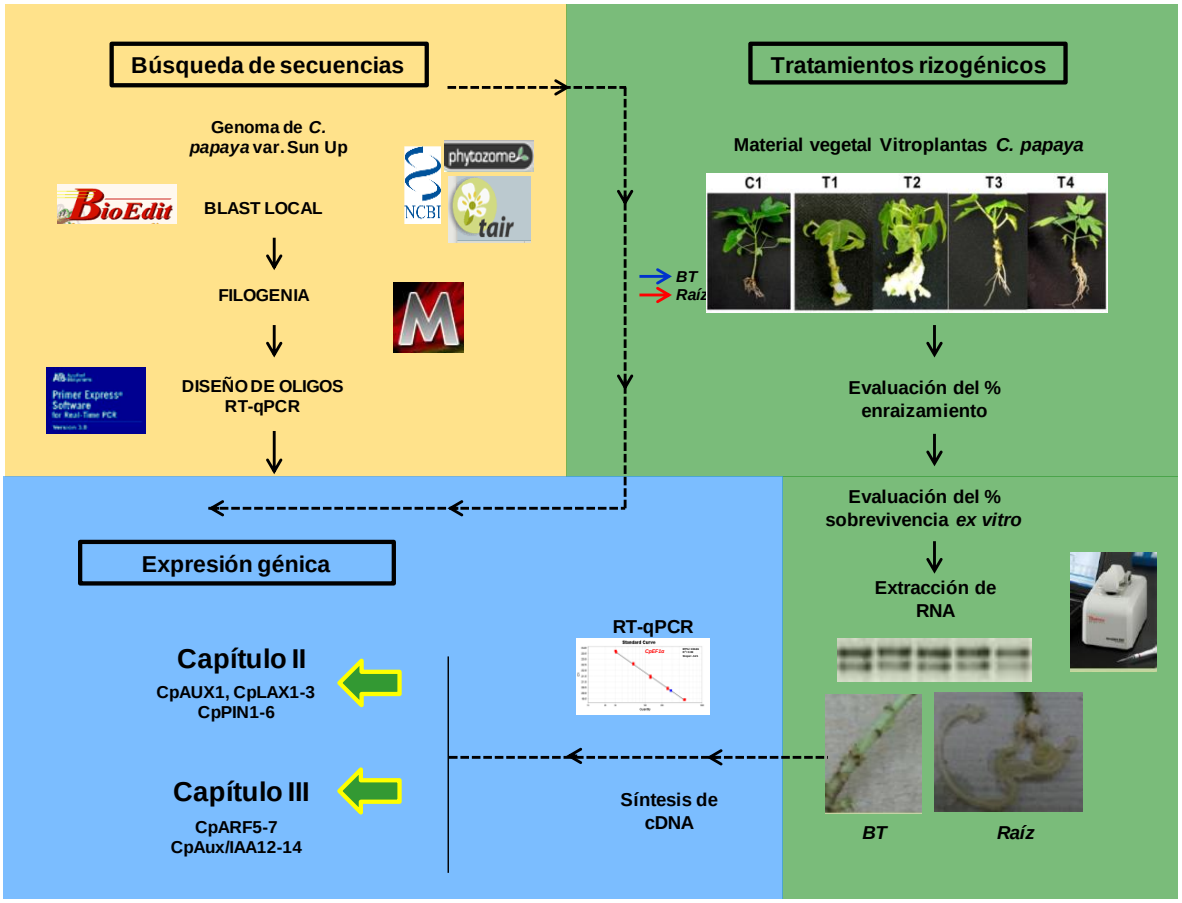
1.3.1. Objetivos específicos

- Caracterizar la estructura y filogenia de genes ortólogos de tipo Aux/IAA-ARFs y AUX1/LAXs-PINs en el genoma secuenciado de *C. papaya* L. cv. Sun Up.

- Obtener *in vitro* plántulas de *C. papaya* cv. Maradol con mejores capacidades rizogénicas

- Análizar la expresión de genes reguladores de la transcripción de auxinas (CpAux/IAA y CpARFs) así como de genes involucrados en el transporte polar de auxinas (CpAUX1/LAXs y CpPINs) en *in vitro* plántulas de *C. papaya* L. cv. Maradol expuestas a diferentes tratamientos rizogénicos.

1.4. ESTRATEGIA EXPERIMENTAL



CAPÍTULO II.

The papaya CpAUX1/LAX and CpPIN genes: structure, phylogeny and expression analysis related to root formation on *in vitro* plantlets [1]

2.1. ABSTRACT

Carica papaya L. plantlets, normally exhibit low rooting capacity when cultured *in vitro*. It has been suggested in other species that auxin concentration in root tissues, is the result of a reflux system driven by auxin influx transporters (AIT; AUX1/LAX) and auxin efflux transporters (AET; PIN), that regulates the mechanism of initiation and development of lateral roots. Therefore, in the present paper, we studied the structure, phylogeny and the expression patterns of the whole family of AIT and AET in *C. papaya*, and their possible relation with the limited capacity to generate adventitious roots of *in vitro* cultured papaya plantlets. We found 4 AUX1/LAX genes (CpAUX1, CpLAX1, CpLAX2, CpLAX3) and 6 PIN genes (CpPIN1, CpPIN2, CpPIN3, CpPIN4, CpPIN5, CpPIN6) within the genome of *C. papaya*. The expression patterns and levels of those genes were studied in stem-base and root tissues from *C. papaya* cv. Maradol plants under four different treatments: (1) *in vitro* plantlets without IBA (that did not generate roots), (2) *in vitro* plantlets treated with 2 mg L^{-1} IBA (that did generate roots), (3) de-rooted seedlings treated with the same concentration of IBA (that also generated adventitious roots), and (4) intact seedlings used as controls. Histological studies made on the stem base and root tissues from all treatments showed that the IBA-induced roots were histologically equivalent to those naturally formed in intact seedlings. *In vitro* plantlets not treated with IBA had low expression of all auxin transporters genes in stem-base tissues and they were unable to produce roots. On the contrary, *in vitro* plantlets treated with IBA experienced a marked increase in the expression of most auxin transporters genes, in particular of CpLAX3 and CpPIN2, and they were capable to produce roots. Those roots generated in the IBA-treated *in vitro* plantlets, showed expression levels and patterns of auxin transporter genes, equivalent to those shown in both, the IBA-treated de-rooted seedlings, and in the naturally formed roots from intact seedlings.

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2.2. INTRODUCTION

The most common form of propagating *Carica papaya* (*C. papaya* L.) is by seeds (Jiménez et al., 2014), however it can also be micropropagated by *in vitro* tissue culture techniques, with the advantage of obtaining genetically homogeneous *in vitro* plantlets (Talavera et al., 2007). Although there are reports of micropropagation of *C. papaya* (Drew and Miller, 1993; Islam et al., 1993; Magdalita et al., 1997; Yu et al., 2000), the *in vitro* rooting and *ex vitro* acclimatization, are fundamental to the establishment of an efficient and low cost large scale micropropagation protocol. Limited rooting in *C. papaya* micropropagated plantlets might result in high (70 %) plant mortality during the *ex vitro* stage, before they can be planted in the field (Malabadi et al., 2011). Therefore, high rooting and efficient acclimatization represent a problem in commercial micropropagation of *C. papaya* cultivars, in particular the cv. Maradol.

The phytohormone auxin, which is synthesized mainly in the shoots and translocated to the sites of action, plays critical roles in regulating plant development and growth (Quint and Gray, 2006; Teale et al., 2008; Hoffmann et al., 2011; Chen et al., 2014; Shen et al., 2015). In the plant, auxins may be distributed in two interconnected transport systems: first, a fast non-directional stream in the phloem along with photosynthetic assimilates, and a second one, that is slow and directional cell-to-cell polar auxin transport (PAT). In this way, PAT distributes auxin in a precise manner that is critically important for the formation of local auxin maxima, mainly in developing tissues (Adamowski and Friml, 2015). Coordinated action of auxin biosynthesis, perception, signaling and PAT are required to form normal lateral roots (Lavenus et al., 2013). In *Arabidopsis thaliana* (*A. thaliana*) the expression of the auxin-responsive reporter gene DR5, decreased after plants were exposed to the auxin efflux inhibitor 1-N-naphthylphthalamic acid, indicating that these auxin responses depend on PAT (Himanen et al., 2002; De Smet et al., 2007; Moreno-Risueno et al., 2010). Ivanchenko et al. (2015) showed that the tomato diageotropica mutant (dgt; deficient of PAT), is not capable to form lateral roots, demonstrating that normal PAT fluxes along the root tip are required for lateral root formation. In roots, the natural auxin indol acetic acid (IAA) moves acropetally (from apical regions to the root apex) and basipetally (from the root apex towards apical zones) by outer layers of the root, and PAT is carried out by the auxin influx (AIT) and auxin efflux (AET) transporter proteins

(Muday and DeLong, 2001). A number of AIT, such as AUX1/LAX (AUX1-like), and AET, such as PINFORMED (PIN) genes, have been isolated and characterized in *A. thaliana* (Vanneste and Friml, 2009; Ugartechea-Chirino et al., 2010).

Within the *A. thaliana* genome, four genes encode for AIT (AUX1, LAX1, LAX2 and LAX3). The size of those genes are: AtAUX1, 4188 pb; AtLAX1, 3942 pb; AtLAX2, 2547 pb; AtLAX3, 2400 pb, while their exons number are: 9, 9, 6 and 7, respectively (NCBI, http://www.ncbi.nlm.nih.gov). The cellular entry of auxins is performed through AUX1 and its orthologs LAX1, LAX2 and LAX3 which encode highly conserved transmembrane proteins with permease activity. AIT proteins (AUX1/LAX) family readily transport auxin across cell membranes (Ugartechea-Chirino et al., 2010) and they are involved in many post-embryonic developmental processes such as: gravitropism, phyllotaxis and lateral root formation (Parry et al., 2001b; Marchant et al., 2002; Reinhardt et al., 2003; Swarup et al., 2005; Dubrovsky et al., 2006; Bainbridge et al., 2008; Laskowski et al., 2008; Swarup et al., 2008). AUX1/LAX proteins, range between 487 and 553 aminoacids (AA) and they show a highly conserved central region with 10 transmembrane helices. LAX proteins are rich in acidic AA in their N-terminal and they are rich in proline at their C-terminal (Shen et al., 2010). In addition, in *A. thaliana*, eight genes of the PINFORMED family (PIN 1 to PIN 8; Gälweiler et al., 1998; Müller et al., 1998) are involved in the facilitation of AET, which is an essential step in PAT (Sawchuk and Scarpella, 2013). These proteins were named after the creation of the first mutant pin1, that develops pin-shaped inflorescences and they are defective in PAT and showed loss of function in the PIN1 gene.

In the present work, we performed an *in silico* characterization of the whole families of AUX1/LAX and PIN in the *C. papaya* genome, and primers were designed for those genes. Experiments were then designed to evaluate different auxin-treatments that allowed efficient adventitious root formation on *in vitro* plantlets of *C. papaya* cv. Maradol versus treatments that did not promote adventitious root formation. The expression patterns of CpAUX1/LAX and CpPIN genes were then analyzed in plantlets under those contrasting treatments, in order to determine if a correlation exists between changes in the expression patterns of CpAUX1/LAX and CpPIN genes, with the capacity of auxin-treated *in vitro* plantlets of *C. papaya*, to form adventitious roots.

2.3. MATERIALS AND METHODS

2.3.1. Plant material

2.3.1.1. Seedlings

Fifty *C. papaya* cv. Maradol seeds were germinated and 2 days after germination, stem base (SB) and root (R) tissues from those seedlings were collected. All seedlings were placed in the greenhouse in nursery plastic trays containing a peat-moss:agrolite (2:1) substrate. Seedlings were watered with 10 mL of sterile distilled water every 48 h, with the addition of 1 g L^{-1} Benlate fungicide. The greenhouse conditions were: temperature (T) of 35 ± 1 °C, relative humidity (RH) of 70 % and PPFD of 750 µmol photon $m^{-2}s^{-1}$.

2.3.1.2. *In vitro* plantlets

Four hundred and twenty *in vitro* shoots originally obtained from 1 year-old plants of *C. papaya* cv. Maradol plants, were cultured *in vitro* inside culture flasks (with three plants each), containing sterilized peat-moss:agrolite (2:1) substrate, 2.21 g L^{-1} MS salts (Murashige and Skoog, 1962), 10 g L^{-1} sucrose, but with 14 different auxin treatments (IBA, NAA or IBA + NAA) in experiment 1 (T1 -T14; Table 2.1), or with (2 g L^{-1}) and without IBA in experiment 2 (D1 - D4; Table 2.2). Shoots from all treatments were cultured in growth rooms (T= 25 °C, PPFD= 300 µmol photon $m^{-2}s^{-1}$ and 12 h photoperiod). At the end of the 21 days exposure to each treatment, plant height, leaves number, root number, root length and rooting percentage were recorded. Then, the plantlets from each treatment were transferred to plastic trays containing peat-moss:agrolite as substrate and 1 g L^{-1} of fungicide (Benlate) and covered with a dome (to maintain a high relative humidity), under greenhouse conditions (T= 35 °C, RH= 70 % and PPFD= 750 µmol photon $m^{-2}s^{-1}$). After growing in the greenhouse for further 42 days, plant survival percentage was evaluated for each treatment.

2.3.1.3. IBA treatment on de-rooted seedlings of *C. papaya*

Fifteen seeds *C. papaya* cv. Maradol were germinated and the resulting intact seedlings were used as controls for anatomical studies and plant height, leaves number, root number, root length and rooting percentage were recorded at day 0 (C1) and 21 days after germination (C2). Another fifteen seedlings were germinated and 2 days after germination, the resulting seedlings were de-rooted (i.e. roots were cut off at the stem-base). The cut end stems of the de-rooted seedlings were rinsed with 10 mL of sterile distilled containing 1 g L^{-1} fungicide (Benlate) and 2 mg L^{-1} IBA was added as adventitious root inducer. Those seedlings were measured 10 days after roots were removed and new adventitious roots were formed (day 0, C3), and 21 days after (C4) (Table 2.2). Seedlings from the four treatments (C1 - C4) were then transferred to plastic trays containing peat-moss:agrolite (2:1) as substrate and 1 g L^{-1} of fungicide (Benlate), under greenhouse conditions (T= 35 ± 1 °C, RH= 70 % and PPFD= 750 µmol photon $m^{-2}s^{-1}$), where the de-rooted seedlings took 10 days to generate new adventitious roots (considered as day 0; C3). After growing for further 21 days under those conditions, previously de-rooted seedlings, now bearing new adventitious roots (C4), were sampled for growth, anatomical studies and gene expression analysis.

2.3.1.4. *In silico* cloning

The *A. thaliana* AUX1/LAX and PIN nucleotide sequences were obtained from Phytozome database (http://phytozome.net), and their corresponding homologous sequences were identified within the genome of *C. papaya* cv. Sun Up. Sorting and identification of those sequences with higher percentage of identity were made with Blast Parser v1.2 program (http://geneproject.altervisa.org). The sequences were selected with the following parameters: coding sequences with E values <10-14, sequences with percentages of identity and similarity greater than 60 % within the genome of *C. papaya* cv. Sun Up. The selected genomic sequences were translated in all six possible reading frames; using the algorithms software of gene prediction FGENESH program (http://linux1.softberry.com/all.htm) and the genetic code of dicotyledonous plants (*A. thaliana*). These predicted protein sequences were edited with the TRANSLATE program with the output format that includes the protein sequence. Using ortholog genes from *C.*

papaya cv. Sun Up, specific oligonucleotides were designed for the qRT-PCR expression analysis of CpAUX1/LAX and CpPIN genes in *C. papaya* cv. Maradol. The oligonucleotides were designed with Primer Premier ver. 6. Program (http://www.premierbiosoft.com/primerdesign/).

2.3.1.5. Protein alignment and phylogenetic analysis

The multiple alignment and phylogenetic trees were constructed using the sequences AtAUX1/LAX and AtPIN proteins from *A. thaliana* and orthologs CpAUX1/LAX and CpPIN proteins from *C. papaya* cv. Sun Up. The phylogeny was obtained by Neighbor-Joining distance based method with 1000 bootstrap replications, and the calculated evolutionary distances were obtained from the Poisson correction evolutionary model, gaps and missing data were treated with pairwise deletion. Edition of the alignment was performed with BioEdit program (Hall, 1999). Both trees were constructed with MEGA 5 program (Tamura et al., 2011). AIT and AET family's members were aligned with ClustalX 1.81 (Blosum Weight Matrix; Gap Opening Penalty: 5; Gap Extension Penalty: 0.20; Thompson et al., 1997).

2.3.1.6. RNA isolation and qRT-PCR

Total RNA was extracted from SB and R tissues from seedlings (intact or de-rooted) and from *in vitro* plantlets, exposed or not to 2 mg L^{-1} of IBA, using the CTAB protocol from Idrovo et al. (2012). The concentration and purity of RNA samples were determined with a Nano-DropTM 1000 Spectrophotometer (Thermo Scientific NanoDrop Technologies, LLC, Wilmington, DE, USA). For first-strand cDNA synthesis, total RNA (5 mg) and 200 U of Superscript III reverse transcriptase were used, following the manufacturer's protocol (InVitrogen/Life Technologies, CA, USA). qRT-PCR was performed in a thermocycler STEP ONE SYSTEM and StepOne Software v2.3 (Applied Biosystems, Foster City, CA, USA). Expression levels were calculated relative to the expression level of *C. papaya* elongation factor 1-α (CpEF1α) using the DDCt method (Applied Biosystems). The specificity of the reactions was confirmed by the standard melt curve method. Each assay was repeated at least three times. Statistical analysis was performed using STATGRAPHICS Plus (http://www.statgraphics.com). One-way analysis of variance

(ANOVA) was used to evaluate the relative expression levels of CpAUX1/LAX and CpPIN genes found in SB and R tissues from plants from the different treatments.

2.3.1.7. Histological analysis

Fresh samples from SB and R tissues from seedlings or *in vitro* plantlets of *C. papaya* cv. Maradol were collected at day 21 of the rooting stage. Subsequently, cross-sections of SB at a distance of 0.2 cm from the roots, and from R at a distance of 0.2 cm from the stem-base were used. All samples from SB and R from seedlings, de-rooted seedlings, and *in vitro* plantlets were treated according to the protocol of Berlin and Miksche (1976). Tissues were infiltrated and embedded in plastic resin (JB-4 Glycol Methacrylate, Polysciences, Angeles, CA, USA). Transverse sections 3 µm thick were cut using a microtome (Microm HM 325; Thermos Scientific, Walldorf, Germany). Sections were stained with Schiff reagent as described by McManus (1961) and 7 % (w/v) Naphthol blue black and mounted in Poly-mount (Polysciences Inc., Warrington, PA, USA). Series of sections from SB and R tissues were then observed and photographed, using a microscope (Axioscope A1, Carl Zeiss, Germany).

2.3.1.8. Statistical analysis

All *in vitro* treatments were analyzed as completely randomized designs. Each experiment was repeated three times using 10 replicate culture flasks per treatment (each with three plantlets). Data were analyzed using one-way ANOVA, and the level of Least Significant Difference was determined using Tukey multiple range test at *$p<0.05$ for comparing treatments means. The program used was STATGRAPHICS Plus (http://www.statgraphics.com) and graphs were performed using the Sigma Plot ver. 11.0 Program.

2.4. RESULTS AND DISCUSSION

Although some species easily form adventitious roots under conditions of *in vitro* culture, for many other species, such as *C. papaya*, the formation of roots under *in vitro* culture conditions is limited (Yu et al., 2000). It is known that auxins and possibly auxin transporters, among other factors, might be involved in the limited capacity to develop roots under *in vitro* culture conditions. In other species such as *A. thaliana*, the expression of auxin transporters genes in roots and leaves have been studied, however, little information on the expression levels of auxin transporter genes is available for tropical species such as *C. papaya*. Particularly, little information is available for gene expression studies of auxin transporter genes in the stems of tissue cultured plantlets. Therefore, it was important to gain understanding on the expression of auxin transporters genes at the stem-base of *C. papaya* plantlets, under conditions that may promote the formation of adventitious roots.

2.4.1. Exogenous auxin promoted adventitious root formation on *in vitro* plantlets of *C. papaya* cv. Maradol

All *in vitro* plantlets at the beginning of the different treatments had an average plant height (PH) of 2.79 cm. At the end of the 21 days exposure to the different treatments, plantlets reached a PH of 3.21 cm, and they had a leaf number (LN) of 4.23, on average. In terms of root number (RN) and root length (RL), when *in vitro* plantlets were not treated with any plant hormone (T1 and T6, Table 2.1), they were not able to generate any adventitious roots, causing high mortality at 42 days after being transferred to the greenhouse. On the contrary, when plantlets were treated with 2 mg L^{-1} IBA (T4), they formed the highest RN (5.78), the highest RL (3.85 cm), the best rooting percentage (96.5 %) and the highest survival *ex vitro* (88.9 %). The addition of NAA alone to the culture medium did result in limited rooting and low survival (T7 - T10, Table 2.1). In combination (IBA + NAA; T11 - T14), plantlets did show intermediate rooting percentage and survival. In summary, these results pointed out that IBA was the best phytohormone to induce root formation and that 2 mg L^{-1} IBA was the ideal concentration to achieve high adventitious rooting in this papaya cultivar. Also, that rooting is critical for *in vitro* plantlets of papaya cv. Maradol, as those treatments with low rooting had very low *ex vitro* survival. On the contrary, plantlets treated

with IBA (T4, Table 2.1), that had the best rooting number and the longest roots, also showed the highest plant *ex vitro* survival.

These results were confirmed in a second experiment, now comparing *in vitro* plantlets (Table 2.2, D1 - D4; Figure 2.1 c, d, g, h) with intact or de-rooted seedlings (Table 2.2, C1 - C4; Figure 2.1 a, b, e, f). Again, these *in vitro* plantlets that were not treated with IBA did not generate roots and exhibited high mortality *ex vitro* (Table 2.2, D2). In contrast, *in vitro* plantlets treated with 2 mg L^{-1} IBA induced high rooting (93.3 %) and exhibited high *ex vitro* survival (90.8 %; Table 2.2, D4). In order to evaluate if the process was also observed under natural, *ex vitro* conditions, seedlings were de-rooted, and the same concentration of IBA (2 mg L^{-1}) was applied to the cut end of the de-rooted seedlings, and their capacity to generate new adventitious roots was evaluated. As shown in Table 2.2, C1- C4 and Figure 2.1 a, b, e, f, intact seedlings (used as controls) at day 21 reached a PH of 17.5 cm, a LN of 10.24, a RN of 16.77, RL of 12.29 cm, 100 % rooting and 100 % survival (Table 2.2, C2; Figure 2.1 e). On the other hand, de-rooted seedlings treated with IBA, showed at day 21, a PH of 8 - 8.9 cm, a LN of 8.52, RN of 14.54, RL of 6.61 cm, high rooting (85.2 %) and high survival (91.1 %) (Table 2.2, C4, Figure 2.1 f).

Our results are consistent with previous reports where the exogenous application of auxins (IBA and NAA) also induced high rooting on *in vitro* papaya shoots of a different cultivar (Drew, 1987). Also Drew et al. (1993) reported that rooting in papaya of different cultivars can be promoted by a short exposure (3 days) to IBA. In other micropropagated species, the use of different concentrations of IBA in the rooting medium of *Rumex palustris* and *Rumex thyrsiflorus* promoted high rooting (Visser et al., 1995).

Table 2.1 Plantlets height (cm), leaves (numbers), root (numbers), root length (cm) and rooting (%) from *in vitro* plantlets of *C. papaya* cv. Maradol treated with different concentrations of IBA, NAA or the combination of both phytohormones.

Condition		Treatment	Height (cm)	Leaves (numbers)	Roots (numbers)	Length of roots (cm)	Rooting at day 21 (%)	Survival at day 42 (%)
IBA	0	T1	$3.15^{b} \pm 0.33$	$4.21^{c} \pm 0.43$	0^{a}	0^{a}	0^{a}	4.3^{a}
(mg L^{-1})	0.5	T2	$3.17^{b} \pm 0.26$	$4.39^{de} \pm 0.38$	$1.22^{e} \pm 0.18$	$0.56^{b} \pm 0.06$	35.6^{d}	23.6^{c}
	1	T3	$3.13^{b} \pm 0.29$	$4.34^{d} \pm 0.45$	$3.17^{f} \pm 0.43$	$2.56^{h} \pm 0.28$	68.8^{f}	65.8^{h}
	2	T4	$3.19^{b} \pm 0.31$	$4.42^{e} \pm 0.39$	$5.78^{j} \pm 0.43$	$3.85^{i} \pm 0.33$	96.5^{i}	88.9^{j}
	3	T5	$3.09^{ab} \pm 0.28$	$4.53^{f} \pm 0.41$	$3.19^{f} \pm 0.39$	$1.55^{e} \pm 0.03$	60.7^{e}	58.1^{g}
NAA	0	T6	$3.04^{a} \pm 0.41$	$3.61^{a} \pm 0.36$	0^{a}	0^{a}	0^{a}	4.3^{a}
(mg L^{-1})	0.5	T7	$3.24^{c} \pm 0.32$	$3.59^{a} \pm 0.32$	$0.12^{b} \pm 0.03$	$0.16^{b} \pm 0.01$	22.2^{b}	15.5^{b}
	1	T8	$2.99^{a} \pm 0.28$	$4.36^{d} \pm 0.41$	$0.35^{c} \pm 0.03$	$0.13^{b} \pm 0.01$	30.3^{c}	17.1^{b}
	2	T9	$3.09^{ab} \pm 0.29$	$4.41^{e} \pm 0.37$	$0.79^{d} \pm 0.07$	$0.75^{c} \pm 0.33$	38.4^{d}	35.5^{e}
	3	T10	$3.05^{a} \pm 0.31$	$4.43^{e} \pm 0.45$	$0.82^{d} \pm 0.09$	$0.88^{c} \pm 0.03$	37.9^{d}	30.4^{d}
IBA+NAA	1 + 1	T11	$3.41^{d} \pm 0.42$	$4.11^{b} \pm 0.41$	$3.32^{g} \pm 0.23$	$1.97^{f} \pm 0.31$	65.9^{ef}	52.8^{f}
(mg L^{-1})	1 + 2	T12	$3.42^{d} \pm 0.35$	$4.21^{c} \pm 0.38$	$3.42^{h} \pm 0.32$	$1.26^{d} \pm 0.01$	70.3^{f}	53.5^{f}
	2 + 1	T13	$3.42^{d} \pm 0.33$	$4.26^{c} \pm 0.47$	$5.35^{i} \pm 0.37$	$2.27^{g} \pm 0.37$	90.7^{h}	82.9^{i}
	2 + 2	T14	$3.38^{d} \pm 0.38$	$4.35^{d} \pm 0.43$	$4.35^{h} \pm 0.37$	$2.06^{f} \pm 0.01$	85.8^{g}	79.1^{i}

Those parameters were taken 21 days after exposure to the corresponding treatment. All the *in vitro* plantlets were transferred to *ex vitro* greenhouse conditions and after 42 days, the survival (%) was evaluated. Data are mean ± SD of three independent experiments, each with three replicates ($n = 3$).

Treatments with the same letters are not significantly different (one-way ANOVA followed by Tukey post-test; * $p < 0.05$).

Table 2.2 Plant height (cm), leaves (numbers), root (numbers), root length (cm) and rooting (%) from *C. papaya* cv. Maradol seedlings and *in vitro* plantlets, evaluated at days 0 and 21.

Plant material	Condition		Treatment	Height (cm)	Leaves (numbers)	Roots (numbers)	Length roots (cm)	Rooting at day 21 (%)	Survival at day 42 (%)
Seedling	2 days after germination	Day 0	C1	$2.4^{a} \pm 0.2$	$3.7^{a} \pm 0.15$	$2.8^{b} \pm 0.23$	$2.5^{c} \pm 0.14$	100^{d}	100^{c}
		Day 21	C2	$14.4^{e} \pm 0.7$	$10.2^{f} \pm .68$	$16.7^{e} \pm 1.3$	$12.2^{f} \pm 0.3$		
		Day 0	C3	$3.4^{c} \pm 0.3$	$4.4^{b} \pm 0.27$	$5.2^{c} \pm 0.28$	$1.3^{b} \pm 0.11$	85.2^{b}	91.9^{b}
Derooted	10 days after germination	Day 21	C4	$8.8^{d} \pm 0.6$	$8.5^{e} \pm 0.24$	$14.5^{d} \pm 1.3$	$6.6^{e} \pm 0.43$		
		Day 0	D1	$3.0^{b} \pm 0.2$	$3.7^{a} \pm 0.18$	0^{a}	0^{a}	0^{a}	5.6^{a}
		Day 21	D2	$3.5^{c} \pm 0.1$	$5.5^{c} \pm 0.25$	0^{a}	0^{a}		
In vitro	IBA (0 mg L^{-1})	Day 0	D3	$3.0^{b} \pm 0.1$	$3.8^{a} \pm 0.21$	0^{a}	0^{a}	93.3^{c}	90.8^{b}
plantlets	IBA (2 mg L^{-1})	Day 21	D4	$3.4^{c} \pm 0.1$	$6.2^{d} \pm 0.21$	$5.4^{c} \pm 0.43$	$4.1^{d} \pm 0.33$		

Those parameters were measured in intact seedlings, IBA treated (2 mg L^{-1}) de-rooted seedlings, *in vitro* plantlets without IBA and IBA-treated (2 mg L^{-1}) *in vitro* plantlets. All the plants, seedlings and *in vitro* plantlets were transferred *ex vitro* under greenhouse conditions and after 42 days, the survival (%) was also evaluated. Data are mean ± SD from three independent experiments, each with three replicates ($n = 3$).

Treatments with the same letters are not significantly different (one-way ANOVA followed by Tukey post-test, * $p < 0.05$).

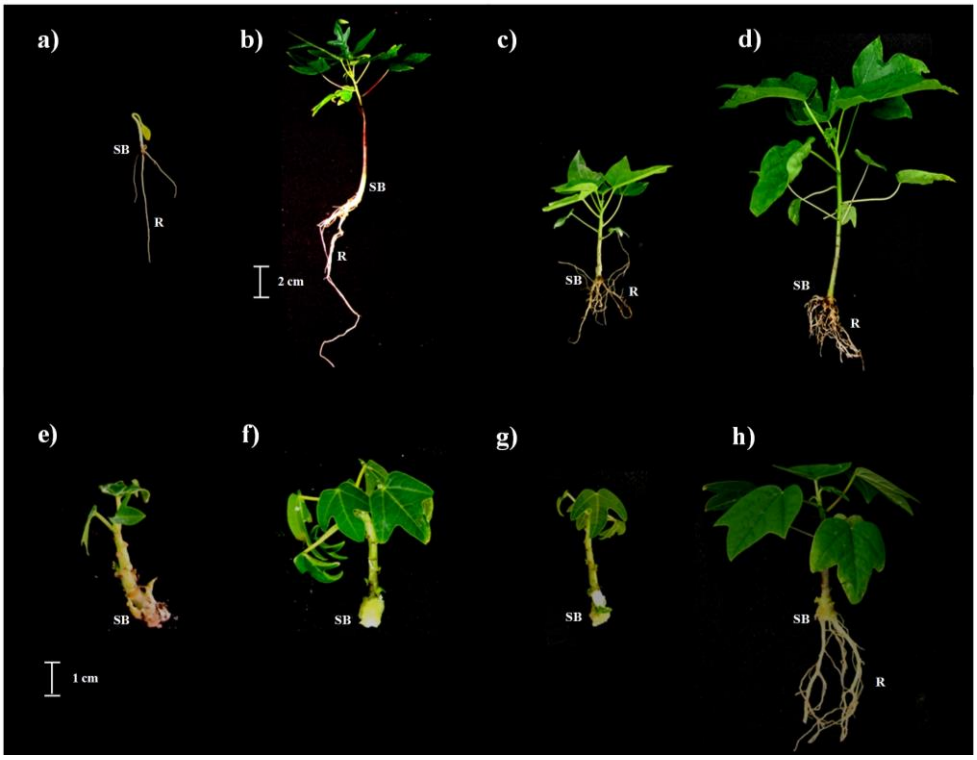

Figure 2.1. Views from intact *C. papaya* cv. Maradol seedlings, 2 days after germination (day 0; **a**) and 21 days after (day 21; **e**); de-rooted seedlings 2 days after germination (day 0; **b**), and 21 days after being treated with 2 mg L^{-1} IBA (day 21; **f**). *In vitro* plantlet not treated with IBA at day 0 (**c**) and at day 21 when no adventitious roots formation occurred (**g**); *in vitro* plantlets treated with exogenous IBA 2 mg L^{-1} at day 0 (**d**) and at day 21 (**h**). The 2 cm scale bar applies for all figures except for **e**, that it has its own scale bar of 15 cm that applies only for **e**.

2.4.2. *In silico* cloning, alignment and phylogenetic analysis of CpAUX1/LAX and CpPIN genes and proteins

Within the *C. papaya* cv. Sun Up genome, we found 4 AUX1/LAX and 6 PIN nucleotide sequences with TBLASTX. All sequences had percentage identities from 66 to 95 % and E value between 7E-11 and 1E-180 (Table 2.3). Both families were classified and named based on the percentage identity and phylogeny with *A. thaliana* AUX1/LAX and PIN genes, a dicot species whose members have all been well characterized at the genomic level. The papaya protein sequences were named as: CpAUX1, CpLAX1, CpLAX2,

CpLAX3 for the AUX1/LAX family and CpPIN1, CpPIN2, CpPIN3, CpPIN4, CpPIN5 and CpPIN6 for the PIN family (Tables 2.3, 2.4).

Table 2.3. BLASTP results of the genomic sequences CpAUX1/LAX and CpPIN genes found in the *C. papaya* cv. Sun Up genome, that show homology to *A. thaliana* AUX1/LAX and PIN genes.

Genes	Segment length (bp)	% Identity	% Similarity	Value E	Score
AUX/LAX					
CpAUX1	1233	95	100	8E-12	233
CpLAX1	1413	95	95	1E-11	232
CpLAX2	1398	95	100	7E-11	176
CpLAX3	1476	86	91	7E-11	194
PINs					
CpPIN1	2270	80	85	1E-175	1330
CpPIN2	1961	67	80	1E-152	600
CpPIN3	2270	66	75	1E-142	685
CpPIN4	2353	91	96	1E-180	1032
CpPIN5	1056	78	92	1E-168	705
CpPIN6	2353	67	79	1E-131	560

In relation to the pair-wise comparison analysis of the predicted protein sequences of AtAUX1/LAX family, CpAUX1 showed the highest percentage identity with AtAUX1 (95 %). CpLAX1 showed a high percentage identity with AtLAX1 (94.06 %), while CpLAX2 showed a 94.3 % of identity with AtLAX2, and finally CpLAX3 presented an 88.1 % of identity in relation to AtLAX3 (Table 2.4). In the PIN family, the AA sequences CpPIN1 reached the highest identity with AtPIN1 (89.08 %), CpPIN2 showed greater identity with AtPIN2 (85 %), CpPIN3 showed an 84.46 % of identity with AtPIN3, similarly CpPIN4 showed higher identity with AtPIN4 (91.95 %), CpPIN5 showed a 69.52 % identity with AtPIN5, whereas CpPIN6 showed the highest percent identity (69.92 %) with AtPIN8.

Table 2.4. Pairwise percentage of identity between auxin transporters of *C. papaya* (CpAUX1/LAXs and CpPIN proteins) and their orthologs in *A. thaliana* (AtAUX1/LAX and AtPIN proteins), the highest percentage is enclosed in italics.

AUX1/LAX	AtAUX1	AtLAX1	AtLAX2	AtLAX3				
CpAUX1	**95.18**	86.11	86.68	77.90				
CpLAX1	93.20	**94.06**	88.38	84.98				
CpLAX2	82.71	88.95	**94.33**	87.81				
CpLAX3	68.92	78.75	84.70	**88.10**				
PIN	AtPIN1	AtPIN2	AtPIN3	AtPIN4	AtPIN5	AtPIN6	AtPIN7	AtPIN8
CpPIN1	**89.08**	79.89	77.01	79.89	52.30	67.24	77.01	45.98
CpPIN2	75.86	**85.06**	78.16	77.01	48.85	63.79	78.16	48.28
CpPIN3	83.91	78.74	**84.46**	79.31	50.00	56.91	80.32	49.43
CpPIN4	79.31	79.31	91.38	**91.95**	50.23	64.37	90.80	47.13
CpPIN5	45.40	49.43	47.70	46.55	**65.52**	40.23	46.55	33.91
CpPIN6	55.75	56.32	56.90	56.32	42.53	51.72	57.47	**69.92**

The AA sequences alignments of the four CpAUX1/LAX proteins (CpAUX1, CpLAX1, CpLAX2 and CpLAX3) are shown in Figure 2.2, while those of the six CpPIN proteins (CpPIN1, CpPIN2, CpPIN3, CpPIN4, CpPIN5 and CpPIN6) are shown in Figure 2.3. The CpAUX1/LAX and CpPIN proteins sequences were analyzed with different sequence multiple alignment and phylogenetic analysis tools, to develop a robust evolutionary model of transporters. Our results, regarding the alignment of AUX1/LAX predicted proteins in *C. papaya* showed an identical profile, length ranged between 387 and 491 AA and high percentage of identity values, equivalent to their counterparts in *A. thaliana*. Therefore, it is likely that CpAUX1, CpLAX1, CpLAX2 and CpLAX3 genes are homologs. In addition, it was also observed the characteristic 10 transmembrane helix regions, acidic AA on its N-terminus and proline-rich AA on its C-terminal; all of these regions were well conserved among all sequences (Figure 2.2).

From the alignment of CpPIN predicted protein sequences, we identified LD proteins (CpPIN 1-4) that showed three TPRXS (N/S) conserved motifs and the hydrophilic loop (crucial to polarity maintenance). The polar location apical-basal of the PIN proteins determines the direction of flow of auxin controlled by the reversible phosphorylation of PIN hydrophilic loops (PINHL; Friml et al., 2004). In addition, two proteins that lack the

TPRXS (N/S) motif and the hydrophilic loop were identified as SD proteins and named as CpPIN5 and CpPIN6 (Figure 2.3).

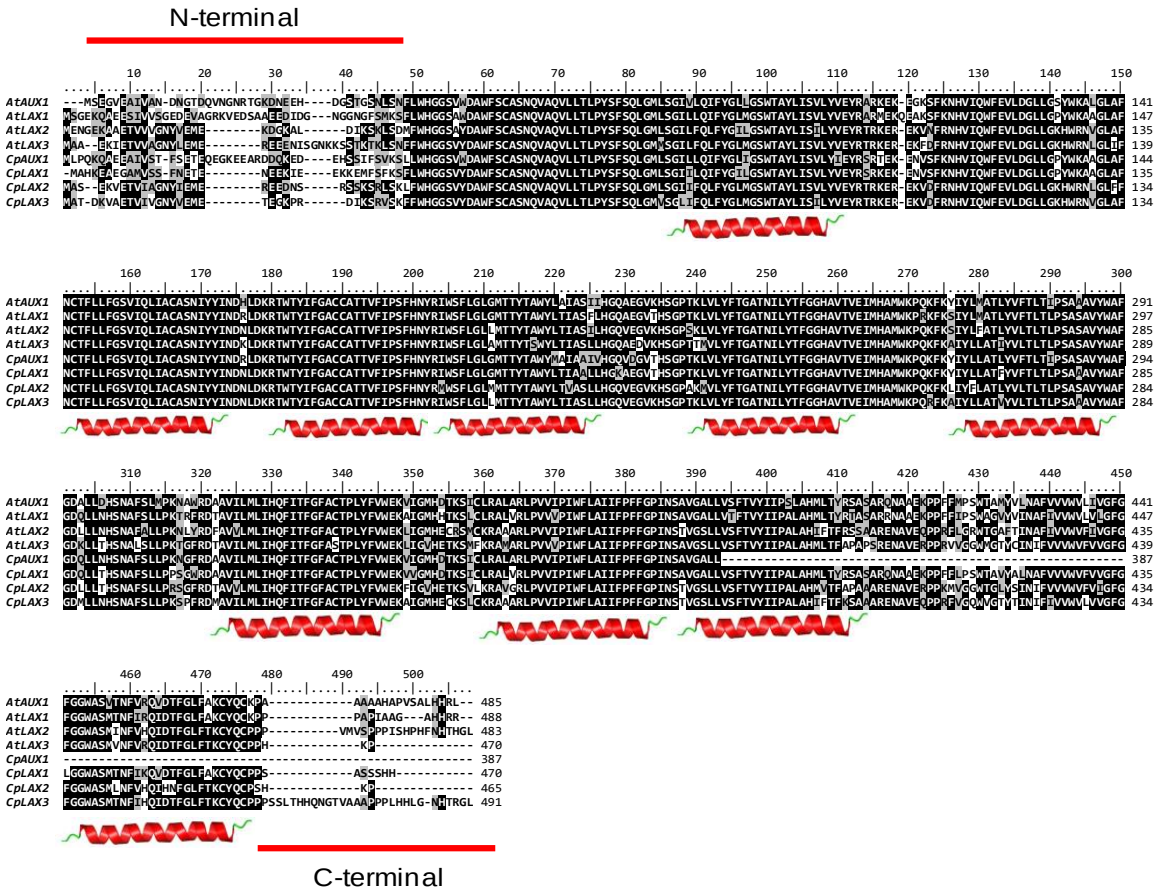

Figure 2.2. ClustalX alignment of the deduced amino acid sequences of *C. papaya* and *A. thaliana* AUX1/LAXs orthologs. Identical amino acids are shaded in black and conservative substitutions are shaded in grey while the other amino acids are different. The regions predicted to form transmembrane helix in the primary structure of CpAUX1/LAXs proteins are marked by red helix. Alignment was made with MEGA5 (Tamura et al. 2011) and edition was made with BioEdit (Hall 1999).

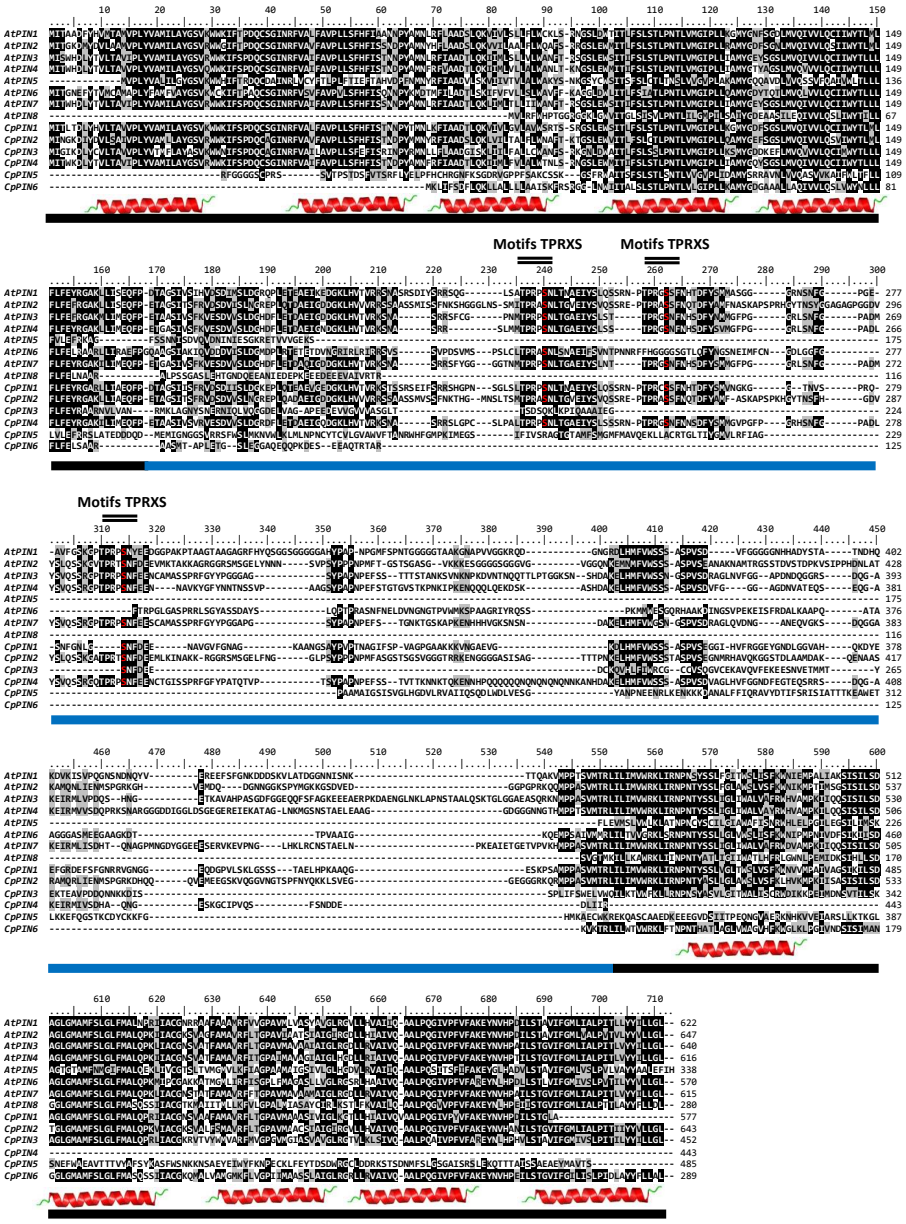

Figure 2.3. Neighbor-joining phylogenetic tree of AUX1/LAX amino acid sequences from *A. thaliana* and *C. papaya* cv. Sun Up showing the formed subclades. AUX1/LAX orthologs sequences from *C. papaya* and *A. thaliana* are highlighted with black and grey circles, respectively. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan and Goldman model (Whelan and Goldman, 2001). The bootstrap consensus tree inferred from 1000 replicates (Saitou and Nei 1987) is taken to represent the evolutionary history of the taxa analyzed (Jones et al., 1992). When the number of common sites was\1000 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. Phylogeny was generated with MEGA5 (Tamura et al. 2011).

Our phylogenetic trees exhibit structural similarities between the predicted proteins AUX1/LAX (Figure 2.4) and PIN (Figure 2.5) from *C. papaya* and those from *A. thaliana*. The phylogenetic tree from CpAUX1/LAX predicted proteins showed two subclades. CpAUX1 and CpLAX1 that were positioned in the same subclade I with their counterparts AtAUX1 and AtLAX1 from *A. thaliana*. Moreover, the proteins CpLAX2 and AtLAX2 are closely related to each other, and for the case of CpLAX3 it shows a high percentage identity (88.10 %) with AtLAX3, these were grouped in the same subclade II (Figure 2.4).

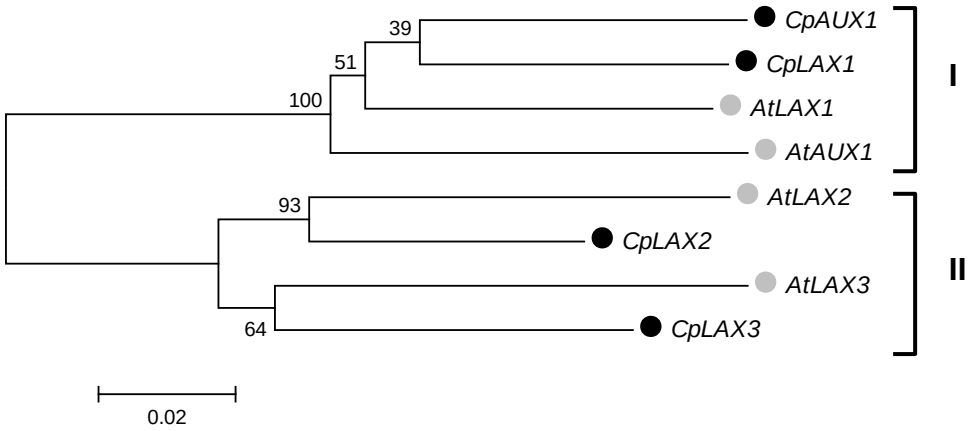

Figure 2.4. ClustalX alignment of the deduced amino acid sequences of *C. papaya* and *A. thaliana* PIN orthologs. Identical amino acids are shaded in black and conservative substitutions are shaded in grey while the other amino acids are different. The double line indicates the three TPRXS from group PIN long-distance, and the central phospho-serines in the motifs are indicated in red. Two hydrophobic domains in the CpPIN proteins are underlined with solid black boxes while the hydrophilic loop region is underlined with solid blue box. The predicted transmembrane helix-formed regions in the primary structure of CpPIN proteins are indicated by red helix. Alignment was made with MEGA5 (Tamura et al. 2011) and edition was made with BioEdit (Hall 1999).

PIN phylogenetic tree showed two subclades, PIN proteins were grouped as follows: subclade I formed by AtPIN1, AtPIN2, AtPIN3, AtPIN4, AtPIN6, AtPIN7, CpPIN1, CpPIN2, CpPIN3, and CpPIN4; subclade II formed by AtPIN5 and CpPIN5, AtPIN8 and CpPIN6. In this sense, CpPIN4 was grouped with its orthologs AtPIN3, AtPIN4 and AtPIN7. In the same way, CpPIN1 was grouped with its ortholog AtPIN1, and CpPIN2 was grouped with AtPIN2, these proteins were found in the same clade, identified as LD transporters. The phylogenetic tree showed that CpPIN5 gene presents high percentage identity (69.52 %) with AtPIN5 gene of *A. thaliana*, also CpPIN6 gene was grouped with its ortholog AtPIN8 gene, these proteins, identified as SD transporters have a hydrophobic conserved domain as in their counterparts for *A. thaliana* (Figure 2.5).

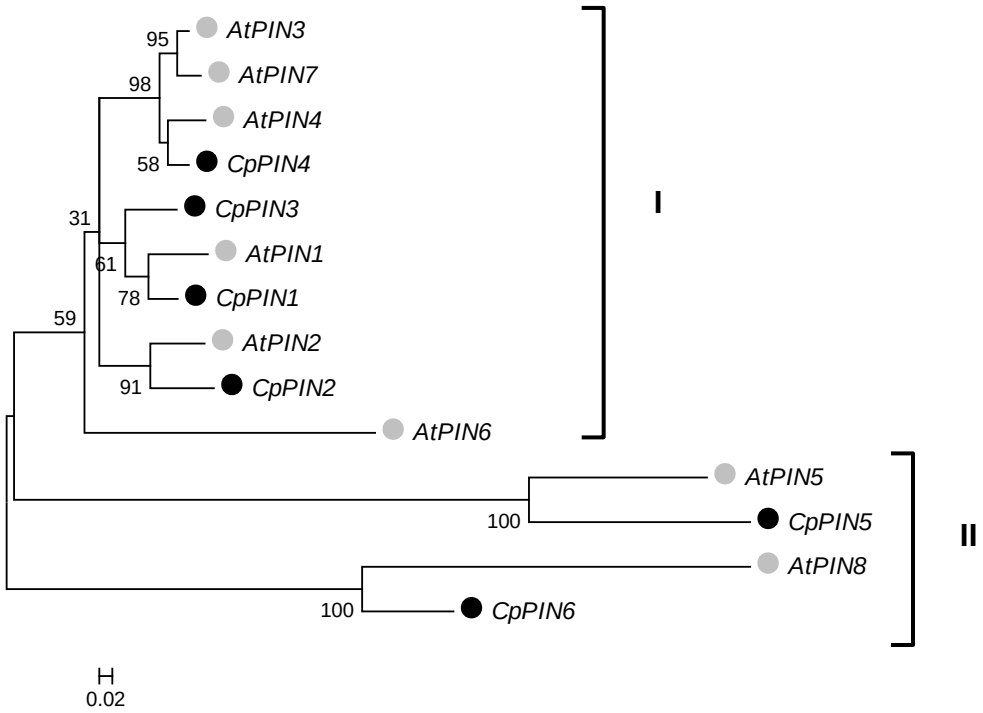

Figure 2.5. Neighbor-joining phylogenetic tree of PIN amino acid sequences from *A. thaliana* and *C. papaya* cv. Sun Up showing the formed subclades. PIN orthologs sequences from *C. papaya* and *A. thaliana* are highlighted with black and grey circles, respectively. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the JTT matrix-based method (Jones et al. 1992) and are in the units of the number of amino acid substitutions per site. Phylogeny was generated with MEGA5 (Tamura et al. 2011).

As mentioned above, auxin molecules can passively enter the cells; however, they can also be transported into cells through the activity of H^+ of the AUX1/LAX family of PM permeases (Kerr and Bennett, 2007). The need for such active auxin uptake processes in the cell causes a high and rapid influx of auxin, as in lateral root cap, where AUX1 plays an important role in the redirection of polar auxin flow (Kramer and Bennett, 2006). The *A. thaliana* genome encodes one AUX1 and three AUX1-like (LAX1, LAX2 and LAX3) proteins that share approximately 80 % similarity in their AA sequences. Therefore, our results agree with those reported by Parry et al. (2001a) and Swarup et al. (2008). Similarly, the gene CpAUX1 and their three homologous CpLAX genes presented between 88 and 95 % similarity at the AA level, suggesting a conserved function in auxin absorption. In *Zea mays*, all four proteins contained a highly conserved core region, which was composed of ten transmembrane helices, the AA composition analysis indicated that C-terminus of the ZmLAX was proline rich and the N-terminus was acidic AA-rich (Yue et al., 2015).

Regarding the PIN proteins, Zažímalová et al. (2007) have found in homologous sequences to those from the model plant *A. thaliana*, relatively high similarity values ranging between 32 and 82 % compared to other higher plants, this suggests that the evolution the PIN arose from a single ancestral sequence (Forestan et al., 2012). From our results, CpPIN proteins showed high homology percentages (values between 66 and 91 %) similarity to those of *A. thaliana* at the AA level that agrees with those previously published. The more significant characteristics of theses CpPIN proteins was the presence of a hydrophilic loop that partially modulates the intracellular auxin homeostasis, depending on cell type and developmental stage, as described by Ganguly et al. (2014). As mentioned by Wabnik et al. (2011) the SD proteins from *A. thaliana* (AtPIN5 and AtPIN8 proteins) showed high homology, these SD proteins appear to be localized at the endoplasmic reticulum (ER), suggesting a possible role in regulating intracellular auxin homeostasis (Wu et al., 2007). The absence of the hydrophilic loop on SD proteins, suggested that there has been no selective advantage to keep this region, whose purpose is crucial to maintain polarity. In our study, we were unable to identify an ortholog for AtPIN6 protein in *C. papaya*. The classification of AtPIN6 protein as LD or SD is rather controversial, since the hydrophilic loop is only partially deduced, while the transmembrane regions show high sequence similarity with LD PINs proteins, as reported

by Křeček et al. (2009) and Mravec et al. (2009). The proteins encoded by these genes can be classified in two groups: (1) PIN LD type proteins (PIN1, 2, 3, 4, and 7) located in the plasma membrane (PM) and involved in processes such as gravitropism, phototropism, embryo development, regulation of root meristem and formation of the apical hook, and (2) PIN SD type proteins (PIN5, 6 and 8) which lack the large hydrophilic loop, these proteins are found in the cytoplasm and the ER (Paponov et al., 2005; Mravec et al., 2009). LD PIN have a hydrophobic region with five to six transmembrane segments (residues 1-163), a predominantly hydrophilic core extending from residue 164-482, and other hydrophobic region with four to five transmembrane segments between residues 483 and 647 (Müller et al., 1998). The hydrophilic loop of PIN1 has three evolutionarily conserved TPRXS motifs that have central phosphorylated Ser residues. These residues are phosphorylated by PINOID (PID), a protein Ser/Thr kinase, whose purpose is to control the polarity of location of PIN genes by direct phosphorylation (Friml et al., 2004; Michniewicz et al., 2007; Fang et al., 2010). Studies in other plant species have revealed the biological functions of auxin transporters (Xu et al., 2014). The evolutionary relationships between *C. papaya* with *A. thaliana* should help us to understand the possible roles of these auxin transporters in *C. papaya*. Another interesting point is that in *A. thaliana*, the AtPIN3, 4 and 7 are closely related to each other, from our results, we also found that CpPIN3 and CpPIN4 showed a high level of similarity and they were grouped in the same clade I (Fig. 5). As it was mentioned by Dal Cin et al. (2009), there are different organizations of the PIN members in different species; this may be due to duplication and specialization of the PIN members in plants. Indeed, as reported by Wang et al. (2009) in rice, the LD OsPIN proteins were grouped with LD proteins AtPIN (AtPIN3, AtPIN4 and AtPIN7) in the same clade. A previous phylogenetic analysis suggested a close evolutionary relationship between these three *A. thaliana* transporters: AtPIN3, AtPIN4 and AtPIN7 that are located in the same clade. The reduction in the number of members for gene families in *C. papaya* in comparison with those in *A. thaliana* has been previously reported for other genes families (Ming et al., 2008; Idrovo et al., 2012; Peraza-Echeverría et al., 2012), and it could be attributed to evolutive differences between *C. papaya* and *A. thaliana* (Ming et al., 2008).

2.4.3. IBA induces changes in the expression of CpAUX1/LAX and CpPIN genes in *C. papaya* cv. Maradol

2.4.3.1. In stem-base tissues

In the case of SB tissues, in general, except for *in vitro* plantlets without IBA, AET genes (dark bars) showed higher relative expression levels (REL) than those of the AIT genes (light bars). Within the auxin AIT genes, CpLAX3 was in general the one showing the highest REL. For the AET genes, CpPIN1 and CpPIN2 were the genes with the highest REL, while CpPIN5 and CpPIN6, had the lowest REL (Figure 2.6). In intact seedlings at day 0, all AIT genes were expressed but only the LD AET (CpPIN1, CpPIN2, CpPIN3, CpPIN4) genes were expressed, whereas the SD AET (CpPIN5 and CpPIN6) genes showed very low REL (Figure 2.6 a). At day 21 however, all AIT and AET genes (including CpPIN5 and CpPIN6) increased their expression, relative to that found at day 0, but they maintained the same expression pattern (Figure 2.6 e). In de-rooted seedlings, at day 0 (taken when the new adventitious roots started to develop, i.e. 10 days after IBA application; Figure 2.6 b), they showed a similar AIT and AET expression patterns to those observed for intact seedlings. At day 21, however, all AIT and AET genes (including CpPIN5 and CpPIN6) increased their REL relative to that found at day 0 (Figure 2.6 f). Again, the expression patterns of those genes found at day 21 in this treatment were similar to those found at the intact seedlings at day 21.

Interestingly, in the case of *in vitro* plantlets not treated with IBA (that did not produce roots), they showed a very low REL of all AIT and AET genes at day 0 (Figure 2.6 c), which remained low at day 21 (Figure 2.6 g). On the contrary, *in vitro* plantlets treated with IBA, at day 0, again the REL of all auxin AIT and AET genes were as low as those found on *in vitro* plantlets without IBA (Figure 2.6 d vs. c), however, at day 21 a marked increase in the REL of those genes occurred (in all except CpPIN 5 and CpPIN 6; Figure 2.6 h). In fact, the expression patterns of those genes for IBA-treated *in vitro* plantlets at day 21 (that did generate roots; Figure 2.6 h), were similar and the REL were as high as those observed at day 21, for both intact seedlings (Figure 2.6 e) and IBA-treated de-rooted seedlings (Figure 2.6 f).

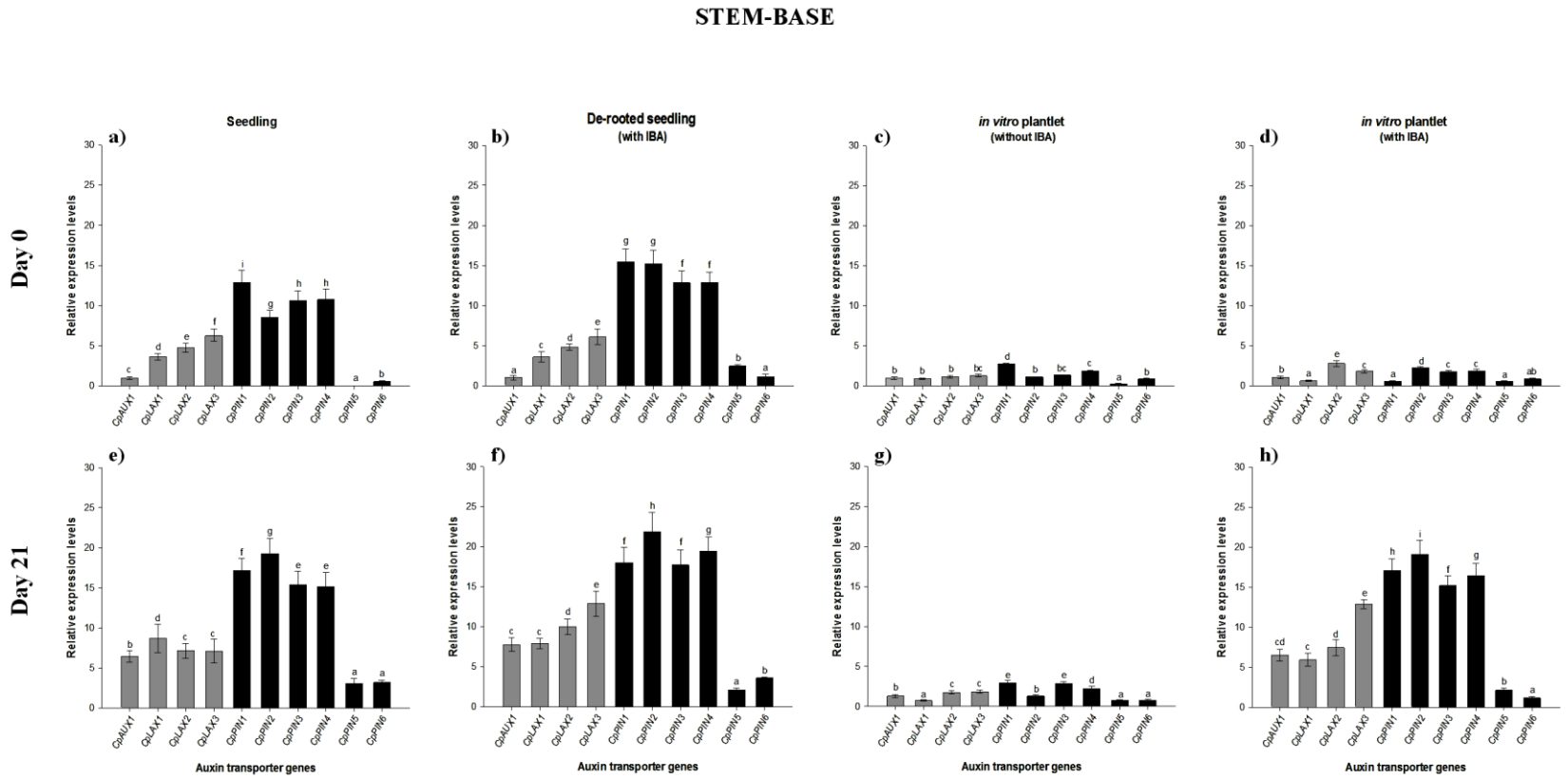

Figure 2.6. qRT-PCR analysis of CpAUX1/LAXs and CpPIN genes expression from SB tissues from intact seedlings, IBA treated de-rooted seedlings, and *in vitro* plantlets of *C. papaya* cv. Maradol. The histograms show the relative expression level of CpAUX1/LAXs and CpPIN genes from seedlings at day 0 (**a**) and at day 21 (**e**), IBA treated de-rooted seedling at day 0 (**b**) and at day 21 (**f**), *in vitro* plantlets without IBA at day 0 (**c**) and day 21 (**g**) and *in vitro* plantlets treated with 2 mg L^{-1} IBA at day 0 (**d**) and at day 21 (**h**). The relative mRNA level of individual genes was normalized with respect to that of CpEF1a gene. The data are mean ± SD from three independent experiments each with three replicates (*n = 3*). Treatments with the same letters are not significantly different (one-way ANOVA followed by Tukey post-test).

2.4.3.2. In root tissues

In root tissues, in general for those treatments that did generate adventitious R, AET genes (dark bars) showed higher REL than those of the AIT genes (light bars), similar to the pattern observed for stem-base tissues. Within the AIT genes, CpLAX3 was in general the one showing the highest REL. For the AET genes, CpPIN2 was the gene with the highest REL, while CpPIN5 and CpPIN6 were the genes with the lowest REL (Figure 2.7). In R tissues from intact seedlings at day 0, all AIT genes were relatively highly expressed. In the case of the LD AET genes (CpPIN1, CpPIN2, CpPIN3, CpPIN4) they were highly expressed, whereas SD AET (CpPIN5 and CpPIN6) showed very low REL (Figure 2.7 a). At day 21, all AIT and AET genes (including CpPIN5 and CpPIN6) increased their REL, relative to that found at day 0 (Figure 2.7 e).

In de-rooted seedlings, at day 0 (taken when the new adventitious roots started to develop, i.e. 10 days after IBA application; Figure 2.7 b), a similar expression pattern of the AIT and AET genes to that observed for intact seedlings occurred. At day 21, again all AIT and AET genes (including CpPIN5 and CpPIN6) increased their REL relative to that found at day 0 (Figure 2.7 f). Again, the expression pattern found at day 21 in this treatment was similar to that found at R tissues from intact seedlings at day 21. In the case of *in vitro* plantlets not treated with IBA, the REL are not available, as this treatment did not produce roots at any time (Figure 2.7 c, g).

The IBA-treated *in vitro* plantlets, at day 0 had no roots (Figure 2.7 d), however, at day 21, this treatment had already generated adventitious roots and interestingly, the expression patterns for both AIT and AET genes (Figure 2.7 h) were in general equivalent to those observed at the same day, for both intact seedlings (Figure 2.7 e) and IBA-treated de-rooted seedlings (Figure 2.7 f). In this treatment, CpLAX3, CpPIN2 and CpPIN4 were the genes with the highest REL (Figure 2.7 h). It is interesting that the REL of CpAUX1 were lower, in this treatment, than those found for this gene in seedlings (Figure 2.7 e) and IBA-treated de-rooted seedlings (Figure 2.7 f).

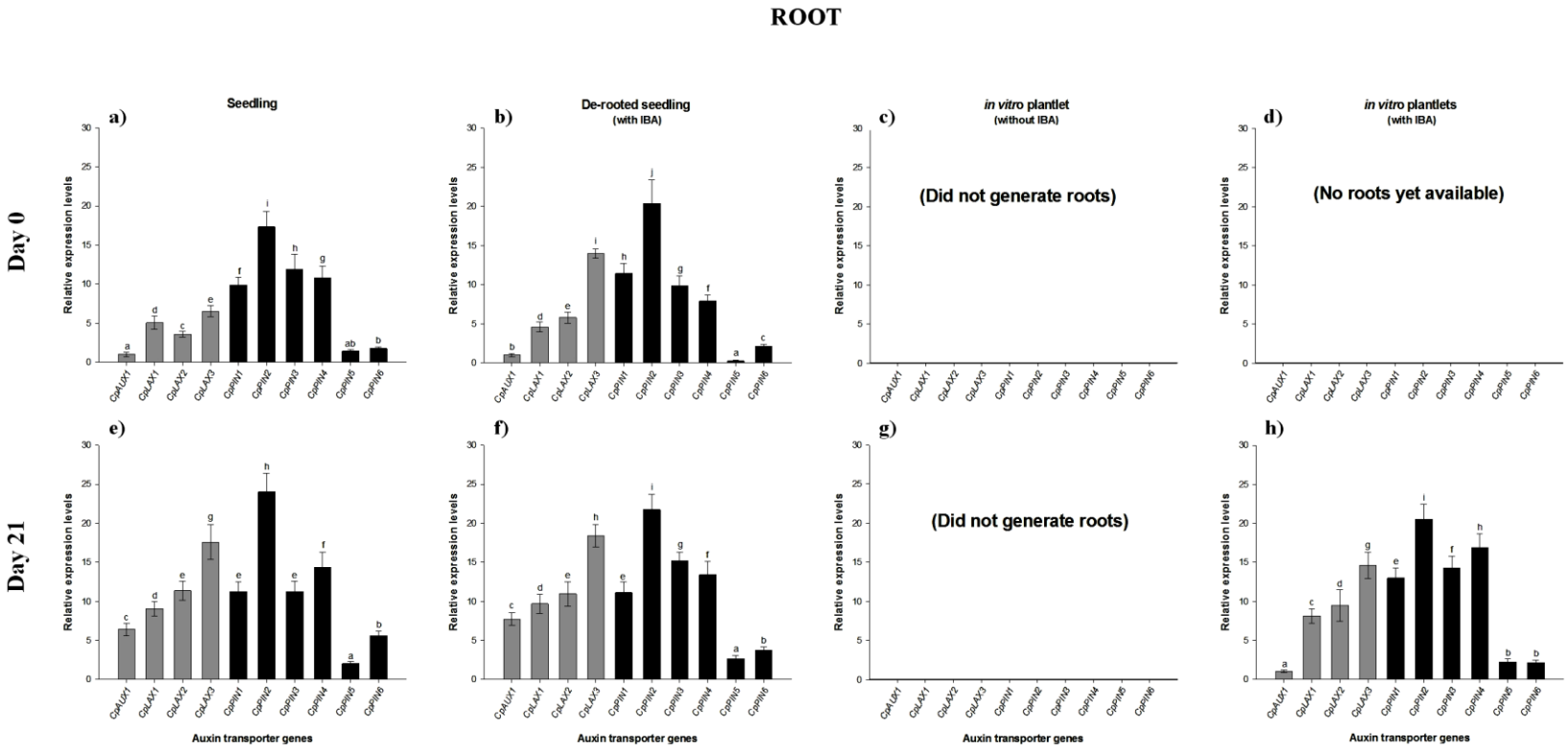

Figure 2.7. qRT-PCR analysis of CpAUX1/LAXs and CpPIN genes expression from R tissues from seedlings, IBA treated de-rooted seedlings and *in vitro* plantlets of *C. papaya* cv. Maradol. The histograms show the relative expression level of CpAUX1/LAXs and CpPIN genes from seedlings at day 0 (**a**) and at day 21 (**e**), IBA treated de-rooted seedling at day 0 (**b**) and at day 21 (**f**), *in vitro* plantlets without IBA at day 0 (**c**) and day 21 (**g**) and *in vitro* plantlets treated with 2 mg L^{-1} IBA at day 0 (**d**) and at day 21 (**h**). The relative mRNA level of individual genes was normalized with respect to that of CpEF1a gene. The data are mean ± SD from three independent experiments each with three replicates (*n = 3*). Treatments with the same letters are not significantly different (one-way ANOVA followed by Tukey post-test).

Auxin transport depends largely on specific auxin transporters, namely the AUX1/LAX and PIN-FORMED (PIN) proteins (Petrásek et al. 2006). Therefore, the expression patterns of AUX1/LAX and PIN genes in seedlings, de-rooted seedlings and *in vitro* plantlets of *C. papaya* cv. Maradol is crucial for understanding the role of IBA in the rhizogenesis processes of this difficult-to-root cultivar.

The large differences in the REL of CpAUX1/LAX and CpPIN genes in both SB and R tissues, between plantlets not treated with IBA and those treated with IBA (that induced high rooting), might indicate that those genes (particularly CpAux3 and CpPIN 1-4) were actively involved in regulating rhizogenesis in *C. papaya*. In our experiments, IBA-treated plants (both *in vitro* plantlets or de-rooted seedlings) CpLAX3 gene had a greater REL compared to the other members of this family. These data are consistent with those reported previously by Swarup et al. (2008), who confirmed that the expression of AtAUX1 and AtLAX3 genes in roots, is a requirement for lateral root development and apical hook formation in *A. thaliana* (Vandenbussche et al. 2010). In addition, it has been observed that defects in AtAUX1 and AtLAX1 genes, reduced initiation and/or elongation of lateral roots due a reduced movement of auxin (Marchant et al. 2002; Benkovà et al. 2003; Wu et al. 2007; Swarup et al. 2008). For instance, aux1 mutants are agravitropic and they have a decreased number of lateral roots. Similar data were observed in loss-of-function mutants for AtLAX3 gen that results in a delayed lateral root emergence (Swarup et al. 2008).

The phenotypes of mutants for the first two PIN genes indicated that PIN-driven PAT is crucial for processes as diverse as aboveground organogenesis and the root gravitropic response (Adamowski and Friml 2015). In seedling, PIN genes maintains activity of the root apical meristem, the local auxin maximum in the root tip is established by directional auxin transport driven by PIN genes and the action of PIN long-distance (LD) establishes a local reflux loop of auxin (Friml and Palme 2002; Blilou et al. 2005). The process in which the root system is formed begins with the emergence of pericycle layer of the primary root, these events are caused in response to high concentrations of auxin involving changes in the distribution of PIN-driven auxin distribution, gradual concentration of auxin at the apical end of the growing lateral root organ (Benková et al. 2003; Dubrovsky et al. 2008; Adamowski and Friml 2015). In *Zea mays* roots, IAA treatment up-regulated the ZmPIN genes expression levels more than five-fold (Yue et al. 2015). From our results, it was

observed that the LD CpPIN1, CpPIN2, CpPIN3 and CpPIN4 genes, increased their expression after 21 days of treatment in all seedlings and de-rooted seedlings in both SB and R tissue. In *in vitro* plantlets, no reports exist on the expression of these auxin genes transporters. However, clearly the *in vitro* plantlets of *C. papaya* need a stimulus for developing roots, this stimulus was achieved by adding IBA to the culture medium, with this condition the *in vitro* plantlets showed a marked increase in the REL of LD CpPIN genes. The increased expression of the CpPIN1, CpPIN2, CpPIN3 and CpPIN4 genes in response to IBA in *C. papaya*, is in line with reports of *A. thaliana* orthologs showing that they are involved in the induction and adventitious root formation (Zažímalová et al. 2010). Regarding the expression of these LD CpPIN genes in other species, Raven (1975), Benková et al. (2003) and Petrásëk et al. (2006), mentioned that AtPIN1, AtPIN2, AtPIN3, AtPIN4, and AtPIN7 genes were detected in the root tip, where they mediate tropism and root patterning, by modulating the maximum auxin concentration and auxin redistribution for root gravitropism.

In our R tissues from seedlings, IBA-treated de-rooted seedlings or IBA-treated *in vitro* plantlets, CpPIN2 gene showed high REL, what is consistent with the fact that in *A. thaliana*, the AtPIN2 transporter has been localized at the apical side in root epidermal cells and at the basal side in young cortex cells, contributing to the reflux of auxin towards to root tip, which is crucial in the control of root meristem size (Blilou et al. 2005). Our results also coincide with the work performed by Křeček et al. (2009), where this gene was found in radicular zones of *A. thaliana*, with absence or low expression in shoot apical areas. Our data confirmed that CpPIN2 gene was highly expressed in both SB and R of those treatments that were able to produce roots, which suggested that this gene may take part in *C. papaya* root architecture. The SD genes, CpPIN5 and CpPIN6 showed low expression in SB and R in all plants evaluated; what might be consistent with the fact that AtPIN8 and AtPIN5 genes of *A. thaliana* had not been reported to influence rhizogenesis, instead they have been expressed in male gametophyte and have a crucial role in pollen development and functionality, their ectopic expression in sporophytic tissues establishes a role in regulating auxin homoeostasis and metabolism (Dal Bosco et al. 2012; Ding et al. 2012).

Interestingly, it seems that CpPIN2 and CpLAX3 genes are directly involved in root formation in *C. papaya*, because they showed higher REL during root formation in seedlings and in de-rooted seedlings, as well as in *in vitro* plantlets after being treated with IBA and formed roots. Other authors have reported that PIN2 and LAX3 for *A. thaliana* (Blilou et al. 2005; Abas et al. 2006; Křeček et al. 2009) and *Medicago truncatula* (Schnabel and Frugoli 2004) are the best candidates for the maintenance of initial PAT in the cambial zone of the roots, therefore is feasible that CpPIN1 and CpPIN2 genes participate in different development processes and show functional redundancy in root zones in plants of *C. papaya*. Most likely, some of these members are also putative regulators and the comparative study of their function and activity will help to clarify their role in the regulation of the direction of PAT of *in vitro* plantlets of *C. papaya*. To understand the biological meaning behind this redundancy, it is necessary to further investigate the different endogenous and exogenous stimuli able to regulate CpAUX1/LAX and CpPIN genes *in vitro* plantlets of *C. papaya* cv. Maradol, to uncover how these auxin transporter genes might participate in PAT.

2.4.4. IBA induced histological changes in SB and R tissues of *in vitro* plantlets, equivalent to those naturally occurring in intact seedlings

2.4.4.1 From SB tissues

Sections from SB from seedlings of *C. papaya* cv. Maradol, showed a mono-stratified epidermis (ep), cortex (co), cambium and strands of xylem (xy) and phloem (ph). In those areas where the root primordia was naturally developing, an irregular arrangement of vv and meristematic zones (mz) can be observed (Figure 2.8 a). In SB sections from IBA-treated de-rooted seedlings, a similar irregular arrangement of vv and mz were observed, in areas where adventitious roots were developing (Figure 2.8 b). On the contrary, SB tissues from the non IBA-treated *in vitro* plantlets did show unaltered arrangement of tissues, and no signs of root primordia were visible (Figure 2.8 c). This unaltered SB histological arrangement, is consistent with the fact that those plantlets were unable to produce roots. However, 21 days after IBA treatment, *in vitro* plantlets suffered marked histological modifications, including the presence of adventitious root primordia (where vv and mz were visible; Figure 2.8 d). SB tissues from those IBA-treated *in vitro* plantlets

were histologically equivalent to those from intact seedlings, bearing naturally formed roots (Figure 2.8 a), and to those IBA-treated de-rooted seedlings developing adventitious roots (Figure 2.8 b).

2.4.4.2. From R tissues

Sections from R tissues from intact seedlings of *C. papaya* cv. Maradol, showed epidermis (e), cortex (co), endodermis (en), perycicle (pe) and strands of ph and xy (Figure 2.8 e). In R sections from IBA-treated de-rooted seedlings, a similar histological arrangement to those described for intact seedlings was observed in the new IBA-induced adventitious R (Figure 2.8 f). On the contrary, non-IBA treated *in vitro* plantlets did not generate R (Figure 2.8 g, empty space). However, the addition of IBA to *in vitro* plantlets resulted in IBA-induced adventitious R, that showed root anatomical features (Figure 2.8 h) that were similar to those observed in R from IBA-treated de-rooted seedlings (Figure 2.8 f), and more importantly to those observed in naturally occurring R from intact seedlings (Figure 2.8 e).

As described by Iliev et al. (2001), auxins might increase the cambial activity and adventitious root formation; therefore tracheid-like cells are formed, providing vascular connection between the root-shoot systems. Our histological observations for *C. papaya* are similar to those reported for Manihot esculenta by Medina et al. (2007), where *in vitro* roots were highly similar to roots from their seedlings (both, in vitro roots and those from seedlings lacked starch granules and had an identical composition and organization). Ballester et al. (1999) also reported the same normal organization of epidermis, cortex and collateral vascular bundles forming a ring around the pith, in roots from *in vitro* plants, and in those from seedlings of *Castanea sativa* Mill.

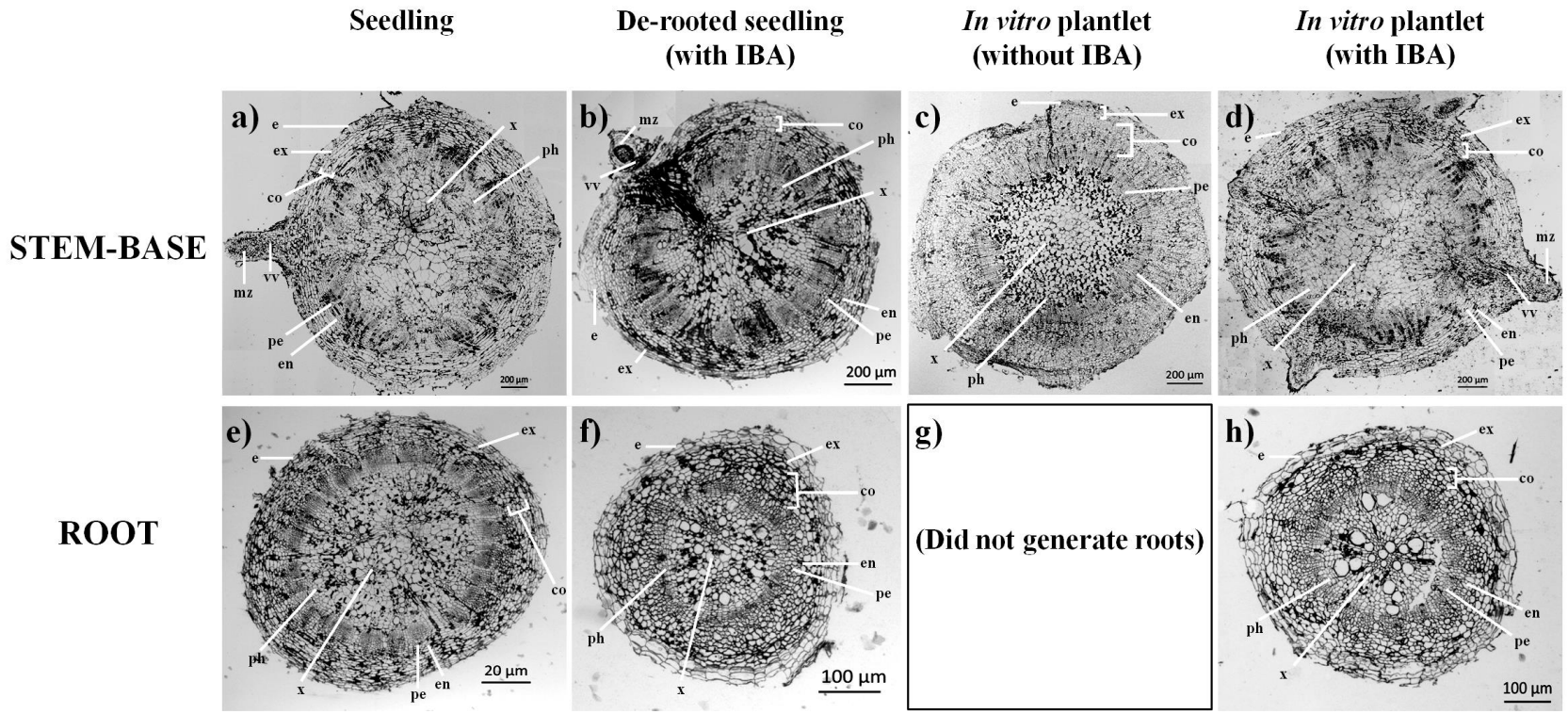

Figure 2.8. Histological studies from SB and R tissues from seedlings, IBA treated de-rooted seedlings, and *in vitro* plantlets of *C. papaya* cv. Maradol with IBA (2 mg L^{-1}) or without IBA. Sections in SB (**a**) and R (**e**) from seedlings, Section in SB (**b**) and R (**f**) from de-rooted seedlings, and cross-sections from SB (**c**) from *in vitro* plantlets without IBA, plantlets in this treatment did not generate roots (**g**). Sections from SB (**d**) and R (**h**) from *in vitro* plantlets treated with 2 mg L^{-1} IBA. Sections were taken 21 days after each treatment was imposed. *e* Epidermis, *ex* exodermis, *co* cortex, *en* endodermis, *pe* pericycle, *ph* phloem, *x* xylem, *mz* meristematic zone, *vv* vascular vessels.

2.5. CONCLUSIONS

We report here the identification, and *in silico* characterization, of the complete family members of AUX1/LAX genes involved in the influx transporters of auxin and of the complete family members of PIN genes involved in the efflux transport of auxins in *C. papaya*. Both families showed high homology with AUX1/LAX and PIN genes from *A. thaliana*. We identified in *C. papaya* the same number of AUX1/LAX genes (four) that those reported for *A. thaliana*, however, in the case of the PIN family members, we identified only six genes from the eight PIN genes reported in *A. thaliana*. Under normal *in vitro* culture conditions, non IBA-treated *C. papaya* plantlets are unable to generate adventitious roots and the expression levels of CpAUX1/LAX and CpPIN genes from their stem base tissues from those plants are extremely low. On the contrary, when exogenous IBA are applied on *C. papaya in vitro* plantlets, they generated adventitious roots (histologically equivalent to those from seedlings), and the expression levels of CpAUX1/LAX and CpPIN genes (in particular CpLAX3 and CpPIN2) from SB and R tissues of those plantlets were reestablished. Both the expression patterns and the REL of those genes at both SB and at the generated R tissues, reached equivalent values to those observed in SB and R tissues from intact seedlings. However, as it has been documented elsewhere that auxin transporters might experience further post-translational regulation (phosphorylation, ubiquitination, and maturation processes, changes in the plasma membrane), that might play an important role in regulating PAT, as suggested by Titapiwatanakun and Murphy (2009). Therefore, the apparent association reported here between the exogenous IBA-induced expressions of those auxin transport genes, with the concomitant exogenous IBA-induced rooting generation, on *in vitro* cultured plantlets of *C. papaya*, merits to be further explored.

CAPÍTULO III.

Identification and characterization of transcription repressors of response to auxin (Aux/IAA) and auxin response factor (ARFs) family genes, related to root formation on *in vitro* plantlets of papaya (*Carica papaya* L.). [2]

3.1. ABSTRACT

Papaya is a species that presents problems during its establishment *ex vitro*, also the *in vitro* plantlets obtained showed low rooting percentages that eventually leads to high mortality when transferred under *ex vitro* conditions. Indole-3-Butyric Acid (IBA) auxin has often been used in culture medium to achieve adventitious root formation in papaya vitroplantlets. Root stimulation is a consequence of auxin regulation by Aux/IAA (Auxin/Indole-3-Acetic Acid) and ARFs (Auxin Response Factors) gene families. When auxin levels are low, Aux/IAA proteins dimerize with ARF activators and thereby repress their activity, in the presence of high auxin levels, Aux/IAA proteins are degraded by SCF^{TIR1} ubiquitin ligase complex, releasing ARFs from this inhibition. In *C. papaya*, the number of Aux/IAA-ARF involved in root formation remains unknown. In this study, a comprehensive characterization and expression profiling analysis was performed of genes involved in the inhibition (CpAux/IAA12, CpAux/IAA13 and CpAux/IAA14) or formation of rhizogenesis (CpARF5, CpARF6 and CpARF7) on *in vitro* plantlets exposed to different rhizogenesis-inducing treatments. In general, *in vitro* plantlets are able to produce adventitious roots when added IBA (2 mg L^{-1}) to the culture medium and by improving *in vitro* conditions such as: increased light intensity (750 µmol photon $m^{-2}s^{-1}$), an adequate supply of sucrose (20 g L^{-1}) and ventilation systems to avoid the accumulation of gases such as ethylene and CO_2, that can inhibit root formation. Our results on expression of Aux/IAA and ARFs genes, suggest that in order for *in vitro* plantlets to be able to produce adventitious roots, they must be exposed to conditions that favor high expression of activators genes (CpARF5, CpARF6 and CpARF7), but low expression of repressor genes (CpAux/IAA12, CpAux/IAA13 and CpAux/IAA14).

[2] **Estrella-Maldonado Humberto**, Talavera May Carlos, Francisco Espadas Gil, Fuentes Ortíz Gabriela, Santana Buzzy Nancy, Saenz Carbonell Luis, Desjardins Yves, Jorge M. Santamaría (2017). Identification and characterization of transcription repressors of response to auxin (Aux/IAA) and auxin response factor (ARFs) family genes related to root formation on *in vitro* plantlets of papaya (*Carica papaya* L.). ***For submitting***

3.2. INTRODUCTION

Auxin biosynthesis and signaling are essential to plant growth and development, and have a key role in vascular differentiation, lateral root formation, tropic responses, shoot elongation and embryo development (Davies, 1995). Therefore, the effects of auxin for plant development including the root formation is through the transcriptional regulation of gene families such as early auxin-responsive, like auxin/indole-3-acetic acid (Aux/IAA) and auxin response factor (ARF) (Shen et al., 2014; Zouine et al., 2014; Liu et al., 2015).

In plants, recent studies have revealed that in absence of auxin, the Aux/IAA proteins interact with its partner ARF, thereby inactivating ARF activity. On the contrary, in presence of auxin, Aux/IAA protein is degraded through ubiquitination by the $SCF^{TIR1/AFBs}$ E3 ubiquitin ligase complex that contains the auxin receptor TIR1/AFBs (Dharmasiri et al., 2005a; 2005b; Kepinski and Leyser, 2005) promoting the de-repression of transcriptional activators ARFs. In *Arabidopsis thaliana* (*A. thaliana*) 29 Aux/IAA proteins and 23 ARF proteins have been characterized (Remington et al., 2004). The function of Aux/IAA and ARF in the regulation of development in *A. thaliana* have been examined through analysis of mutants (Reed, 2001), in specific, Aux/IAA and ARF mutants modules the development and formation of lateral roots (Goh et al., 2012). In *A. thaliana*, Aux/IAA12 and Aux/IAA13 repressor genes inhibit the formation of lateral roots in wild-type plants (Wilmoth et al., 2005), while that the gain-of-function *slr-1* mutation in the *Aux/IAA14* gene blocks pericycle cell division for lateral roots initiation, resulting in a solitary-root phenotype (Fukaki et al., 2002; Vanneste et al., 2005). On the contrary, the loss function of ARF5, ARF7 and ARF19 mutants leads to reductions in the number of lateral roots and in a drastic delay in the formation of lateral roots (Okushima et al., 2005; Wilmoth et al., 2005), also, the double mutation, *arf7/arf19* affects auxin mediated lateral root development (Narise et al., 2010). Furthermore, the overexpression of ARF17 genes leads to increased number of lateral roots and primary root growth in *A. thaliana* (Mallory et al., 2005; Wang et al., 2005).

Papaya (*C. papaya* L.) belongs to family Caricaceae is of the economically important fruits that is distributed has in South Mexico and/or in Central America (Fuentes and Santamaría, 2014). At present, globally Mexico is the fifth largest producer of *C. papaya* fruit and principal exporter of this fruit (FAOSTAT, 2015). To renew and establish new

plantations, techniques *in vitro* micropropagation have been development to obtain high yielding, diseased free genetically-homogeneous elite plants (Talavera et al., 2007). *C. papaya* plants grown under *in vitro* conditions in non-ventilated containers generally have limited photosynthetic capacity because of low CO_2 concentrations and low PPFD. Besides these *in vitro* plantlets usually contain sucrose in their culture medium as the main source of energy, which causes the development of heterotrophic nutrition, however it is desirable that *in vitro* plantlets develop a photoautotrophic nutrition that would be beneficial to obtain a better yield in the acclimatization phase of the plants. Therefore in our study a beneficial alternative for the acclimatization of the *in vitro* plantlets *C. papaya* is the use of atmospheres enriched in CO_2 (ventilated systems) and superior PPFD (high light intensity), while reducing the sucrose of the culture medium. Another critical aspect affecting the *C. papaya in vitro* plantlets is the difficulty to generate an efficient root system (Yu et al., 2000), this hinders plant recovery once planted to the field (Malabadi et al., 2011). To overcome the poor rooting in this species, Indole-3-butyric acid (IBA) is used to induce roots on *in vitro* plantlets (Drew et al., 1993; Estrella-Maldonado et al., 2016).

Taking into account that *in vitro* plantlets of *C. papaya* present an intermediate rooting capacity affecting their survival rates changes in the rooting protocol that is changing light intensity, concentrations of sucrose and *in vitro* ventilation may have beneficial effects on the auxin transport and regulation to better rooting. This may thereby improve regrowth and survival of plantlets upon transfer to soil during acclimatization. Therefore, the present study aim to understand how the expression of genes involved in the transcription of auxin (repressor Aux/IAA and activators ARFs genes) is affected by different rhizogenesis treatments on *in vitro* plantlets of *C. papaya* cv. Maradol.

3.3. MATERIALS AND METHODS

3.3.1. Plant material

3.3.1.1. Seedlings and de-rooted seedlings

Thirty-five *C. papaya* cv. Maradol seeds were germinated under *in vitro* conditions, the seeds were rinsed with 10 mL of sterile distilled containing 1 g L^{-1} fungicide (Benlate) and subsequently cultured *in vitro* inside culture flasks (nine seeds each) containing sterilized peat-moss:agrolite (2:1) substrate and 1.1 g L^{-1} MS salts (Murashige and Skoog, 1962). After of 8 days the seedlings germinated and this was considered as day 0. On the other hand, thirty-five *C. papaya* cv. Maradol seeds were germinated and 8 days after germination the resulting seedlings were de-rooted, i.e. roots were cut off at the stem-base, the cut end stems of the de-rooted seedlings were again rooted with 2 mg L^{-1} IBA to induce adventitious roots. New adventitious roots formed after of 10 days on these de-rooted seedlings. All plants were cultured in growth rooms with T = 25 ºC, PPFD = 200 µmol photon $m^{-2}s^{-1}$ and 12 h photoperiod supplied by white fluorescent lamps.

3.3.1.2. *In vitro* plantlets

Axillary buds (treatments plants) of 0.53 ± 0.23 cm were obtained previously from one-year-old hermaphrodite *C. papaya* cv. Maradol plants. The axillary buds introduced *in vitro* were cultured in 4.43 g L^{-1} MS salts (Murashige and Skoog, 1962), 20 g L^{-1} sucrose, 0.5 mg L^{-1} of kinetin, 0.2 mg L^{-1} 6-benzylaminopurine (BAP), 8 g L^{-1} of agar, pH of 5.8 and subcultured 3 times every 21 days (63 days). After of 3 subcultures the *in vitro* plantlets obtained showed a greater height to 3 ± 0.4 cm and 4-6 leaves of 8 mm. All plants were cultured in growth rooms with T = 25 ºC, PPFD = 200 µmol photon m^{-2} s^{-1} and 12 h photoperiod supplied by white fluorescent lamps.

3.3.2. Rooting conditions cultured

Seedlings, de-rooted seedling (control plants) and *in vitro* plantlets of *C. papaya* cv. Maradol were exposed under *in vitro* conditions in three treatments of sucrose, light and

ventilation. All the plants used in these three treatments were cultured during 42 days (2 subcultured) in their respective treatment inside culture flasks (nine plants each) containing sterilized peat-moss:agrolite (2:1) substrate, 2.21 g L^{-1} MS salts (Murashige and Skoog, 1962), varying sucrose concentration (Sucrose treatment) and different light intensities (Light treatment) and exposed to various ventilation conditions (Ventilation treatment), supplemented with presence or absence of IBA at concentration of 2 mg L^{1}. All plants were cultured in growth rooms with T = 25 ºC, PPFD= 300 µmol photon m^{-2} s^{-1} and 12 h photoperiod. Control plants and *in vitro* plantlets were transferred to acclimatization in plastic trays containing peatmoss:agrolite (2:1), 1 g L^{-1} of fungicide (Benlate) and covered with plastic cover to maintain a high relative humidity (RH), were transferred under greenhouse conditions (T = 35 ± 1 ºC, RH = 70 % and PPFD = 750 µmol photon m^{-2} s^{-1}). After growing in the greenhouse for further 42 days, plant survival percentage was evaluated for each treatment.

3.3.2.1. Sucrose treatment

The following treatments were named as: S1 (seedlings without sucrose and without IBA), S2 (seedlings with 20 g L^{-1} of sucrose and without IBA), S3 (de-rooted seedlings without sucrose and with IBA), S4 (de-rooted seedlings with 20 g L^{-1} of sucrose and with IBA), S5 (*in vitro* plantlets without sucrose and without IBA), S6 (*in vitro* plantlets with 20 g L^{-1} of sucrose and without IBA), S7 (*in vitro* plantlets without sucrose and with IBA) and S8 (*in vitro* plantlets with 20 g L^{-1} of sucrose and with IBA). All the plants were exposed to T = 25 ºC without ventilation system.

3.3.2.2. Light treatment

The following treatments were named as: L1 (seedlings with 200 µmol photon m^{-2} s^{-1} at 25 °C without IBA), L2 (seedlings with 750 µmol photon m^{-2} s^{-1} at 25 °C without IBA), L3 (de-rooted seedlings with 200 µmol photon m^{-2} s^{-1} at 25 °C with IBA), S4 (de-rooted seedlings with 750 µmol photon m^{-2} s^{-1} at 25 °C with IBA), L5 (*in vitro* plantlets with 200 µmol photon m^{-2} s^{-1} at 25 °C without IBA), L6 (*in vitro* plantlets with 750 µmol photon m^{-2} s^{-1} at 35 °C without IBA). L7 (*in vitro* plantlets with 200 µmol photon m^{-2} s^{-1} at 25 °C with IBA), L8 (*in*

vitro plantlets with 750 µmol photon m^{-2} s^{-1} at 35 °C with IBA). All the plants were exposed without sucrose concentrations and without ventilation system.

3.3.2.3. Ventilation treatment

The following treatments were named as: V1 [seedlings in culture flasks covered (without filter) without IBA], V2 [seedlings in culture flasks with plastic lid pierced with a diameter of 1 cm^2 (with filter) without IBA], V3 (de-rooted seedlings without filter and with IBA), V4 (de-rooted seedlings with filter and with IBA), V5 (*in vitro* plantlets without filter and without IBA), V6 (*in vitro* plantlets with filter and without IBA), V7 (*in vitro* plantlets without filter and with IBA) and V8 (*in vitro* plantlets with filter and with IBA). All the plants were exposed without sucrose concentrations and T = 25 ºC.

3.3.3. *In silico* identification of CpAux/IAA and CpARFs proteins

C. papaya Aux/IAA and ARFs sequences were obtained from Plant Transcription Factor database (PlnTFDB 3.0) - Universitaet Potsdam (http://plntfdb.bio.uni-potsdam.de/), however the information obtained in PlTFDB was verified using to blast of *A. thaliana* Aux/IAA and ARFs against the *C. papaya* genome database on Phytozome 10.1 (http://phytozome.net) with TBLASTN.

3.3.4. Protein alignment and phylogenetic analysis

Multiple sequence alignment and phylogenetic trees were constructed using the 18 CpAux/IAA sequences and 12 CpARFs sequences of *C. papaya* compared with 29 Aux/IAA sequences and 23 ARFs sequences identified in *A. thaliana*. Edition of the alignment was performed with BioEdit program (Hall, 1999) with ClustalX 1.81 (Blosum Weight Matrix; Gap Opening Penalty: 5; Gap Extension Penalty: 0.20; Thompson et al., 1997). Both trees were constructed with MEGA 5.1 program (http://www.megasoftware.net/) (Tamura et al., 2011) employing the neighbor joining (NJ) method. Bootstrap values were calculated using 1000 replications and the calculated evolutionary distances were obtained from the Poisson correction evolutionary model, gaps and missing data were treated with pairwise deletion.

3.3.5. RNA isolation and RT-qPCR

Total RNA was extracted from stem-base (SB) and root (R) tissues from seedlings, de-rooted seedlings and *in vitro* plantlets exposed in the treatments different (Sucrose, Light and Ventilation treatments) with or without 2 mg L^{-1} using the CTAB protocol establish by Idrovo et al. (2012). DNase I was used to remove any genomic DNA contamination from total RNA. For first-strand cDNA synthesis 5 mg of RNA and 200 U of Superscript III reverse transcriptase were used following the manufacturer's protocol (Invitrogen/Life Technologies, CA, USA). RT-qPCR analysis was performed using Elongation Factor 1-α (CpEF1α) gen as an internal standard to calculate the relative fold differences based on the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) values as was described by Estrella-Maldonado et al. (2016). The specificity of the reactions was confirmed by the standard melt curve method. PCR conditions were as follows: 95 °C for 10 min, 38 cycles at 95 °C for 15 s, and 60 °C for 60 s. Expression analysis was carried out using five biological repeats and one-way analysis of variance (ANOVA) was performed using Statgraphics Plus (http://www.statgraphics.com). The graphics were performed using the Sigma Plot ver. 11.0. Program.

3.4. RESULTS

3.4.1. Rooting-inducing treatments on *in vitro* plantlets of *C. papaya*

After 42 days of culture, seedlings from S1 and S2 treatments had more roots (12), with average length of 11.5 cm. *In vitro* plantlets from S5 and S6 treatments did not generate roots due to absence of IBA in the culture medium; consequently they displayed high mortality. In contrast, *in vitro* plantlets exposed to IBA (S7 and S8 treatments) were able to generate roots, but they still presented high mortality rates (close to 60 %). All seedlings rooted and consequently all survived after 42 days. In contrast, de-rooted seedlings with or without sucrose, but with exogenous IBA, had an 83 % survival rate (Table 3.1).

Seedlings (L2 treatment) and de-rooted seedlings (L4 treatment) exposed to high light intensities had more roots with lengths of 12.3 and 9 cm, respectively. In contrast, seedlings (L2 treatment) and de-rooted seedlings (L3 treatment) exposed to low light intensities had fewer shorter roots (Table 3.1). *In vitro* plantlets without exogenous IBA, and with low light intensities were not able to generate adventitious roots (L5 treatment), and thus caused high mortality. The *in vitro* plantlets of L8 treatment were the only ones to display high rooting (89.8 %) and high survival (87.4 %). Interestingly, *in vitro* plantlets cultured without IBA in their culture medium but provided with high light intensity (L6 treatment) were able to form roots, and they also survived well (60 %) (Table 3.1).

With respect to ventilation treatments, the seedlings from V1 and V2 treatments, showed significant differences in roots number (11.57 cm for V1 and 11.41 for V2 treatment), in both treatments the roots were 11 cm, these seedlings also showed high survival (Table 3.1). De-rooted seedlings exposed to ventilation system (V4 treatment; exposed with filter), similarly to what it was observed in seedlings, also showed more roots 12.4 longer 9.9 cm. *In vitro* plantlets grown in ventilated vessels (perforated lids cover with filter on the top of the culture flasks) and exposed to exogenous IBA (V8 treatment) they generated 5.28 roots of 3.56 cm in length and showed high rooting percentages (82.9 %) and high survival (81.9 %). In contrast *in vitro* plantlets exposed to exogenous IBA, but without ventilation system, were still able to produce 1.87 roots; these *in vitro* plantlets reached 50 % of rooting and 37 % of survival (V7 treatment) (Table 3.1).

Table 3.1. Plant height (cm), leaves (numbers), roots (numbers), root length (cm) and rooting (%) from seedlings, de-rooted seedlings (control plants) and *in vitro* plantlets of *C. papaya* cv. Maradol exposed in treatments different (Sucrose, Light and Ventilation) to inducing the rhizogenesis.

Treatment		Condition	IBA (mg L^{-1})	Plant height (cm)	Leaves (number)	Roots (number)	Roots lenght (cm)	Rooting at day 21 (%)	Survival at day 42 (%)
		Sucrose (mg L^{-1})							
Seedling	S1	0	0	10.34 ±0.98 ^{d}	10.36 ±0.86 ^{e}	12.23 ±1.28 ^{e}	11.51 ±0.76 ^{d}	100 ^{d}	100 ^{d}
	S2	20	0	10.39 ±1.09 ^{d}	10.95 ±1.06 ^{f}	12.18 ±1.19 ^{e}	11.57 ±0.98 ^{d}	100 ^{d}	100 ^{d}
De-rooted seedling	S3	0	2	8.54 ±0.58 ^{b}	8.01 ±0.32 ^{c}	9.38 ±1.28 ^{d}	7.95 ±0.52 ^{c}	88.8 ^{c}	83.3 ^{c}
	S4	20	2	9.19 ±0.63 ^{c}	8.94 ±0.67 ^{d}	9.45 ±1.19 ^{d}	8.01 ±0.47 ^{c}	88.8 ^{c}	83.3 ^{c}
***In vitro* plantlets**	S5	0	0	3.25 ±0.26 ^{a}	3.21 ±0.33 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}
	S6	20	0	3.34 ±0.16 ^{a}	3.34 ±0.28 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}
	S7	0	2	3.37 ±0.28 ^{a}	3.53 ±0.38 ^{a}	1.11 ±0.09 ^{b}	1.35 ±0.03 ^{b}	50.7 ^{b}	38.4 ^{b}
	S8	20	2	3.42 ±0.22 ^{a}	6.03 ±0.54 ^{b}	2.12 ±0.16 ^{c}	1.45 ±0.06 ^{b}	53.2 ^{b}	39.5 ^{b}
		Light (µmol m^{-2} s^{-1})							
Seedling	L1	200	0	10.23 ±0.67 ^{c}	10.17 ±0.57 ^{e}	11.98 ±1.02 ^{g}	11.31 ±0.64 ^{f}	100 ^{f}	100 ^{g}
	L2	750	0	11.31 ±0.74 ^{d}	14.34 ±1.09 ^{g}	12.74 ±0.92 ^{h}	12.39 ±0.72 ^{g}	100 ^{f}	100 ^{g}
De-rooted seedling	L3	200	2	9.13 ±0.68 ^{b}	8.24 ±0.44 ^{d}	9.41 ±0.52 ^{e}	7.73 ±0.56 ^{d}	88.8 ^{d}	83.3 ^{d}
	L4	750	2	10.37 ±0.32 ^{c}	11.65 ±0.89 ^{f}	10.69 ±0.43 ^{f}	9.01 ±0.62 ^{e}	96.4 ^{e}	93.3 ^{f}
***In vitro* plantlets**	L5	200	0	3.24 ±0.32 ^{a}	3.15 ±0.32 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}
	L6	750	0	3.45 ±0.12 ^{a}	3.98 ±0.31 ^{b}	3.03 ±0.35 ^{c}	2.53 ±0.36 ^{c}	60.5 ^{c}	58.3 ^{c}
	L7	200	2	3.32 ±0.23 ^{a}	3.28 ±0.39 ^{a}	2.02 ±0.15 ^{b}	1.54 ±0.36 ^{b}	52.1 ^{b}	35.4 ^{b}
	L8	750	2	3.57 ±0.27 ^{a}	4.79 ±0.44 ^{c}	4.77 ±0.43 ^{d}	2.49 ±0.36 ^{c}	89.8 ^{d}	87.4 ^{e}

Continuation of ***Table 3.1.***

Treatment		Condition	IBA (mg L^{-1})	Plant height (cm)	Leaves (number)	Roots (number)	Roots lenght (cm)	Rooting at day 21 (%)	Survival at day 42 (%)
		Ventilation							
Seedling	V1	-	0	10.12 ±0.58 ^{e}	9.88 ±0.57 ^{d}	11.57 ±0.81 ^{e}	11.14 ±0.43 ^{f}	100 ^{f}	100 ^{e}
	V2	+	0	10.99 ±0.63 ^{f}	10.41 ±0.69 ^{e}	11.41 ±0.76 ^{e}	11.29 ±0.51 ^{f}	100 ^{f}	100 ^{e}
De-rooted seedling	V3	-	2	9.28 ±0.39 ^{d}	10.21 ±0.83 ^{e}	10.92 ±0.72 ^{d}	8.01 ±0.48 ^{d}	88.8 ^{d}	81.3 ^{c}
	V4	+	2	11.43 ±0.88 ^{g}	11.32 ±0.65 ^{f}	12.41 ±1.15 ^{f}	9.96 ±0.24 ^{e}	95.6 ^{e}	90.3 ^{d}
***In vitro* plantlets**	V5	-	0	3.35 ±0.24 ^{a}	3.33 ±0.24 ^{a}	0 ± ^{a}	0 ^{a}	0 ^{a}	0 ^{a}
	V6	+	0	4.03 ±0.38 ^{b}	5.38 ±0.48 ^{b}	0 ± ^{a}	0 ^{a}	0 ^{a}	0 ^{a}
	V7	-	2	3.12 ±0.29 ^{a}	3.49 ±0.34 ^{a}	1.87 ±0.12 ^{b}	1.22 ±0.11 ^{b}	50.8 ^{b}	37.3 ^{b}
	V8	+	2	4.89 ±0.41 ^{c}	7.56 ±0.56 ^{c}	5.28 ±0.66 ^{c}	3.56 ±3.06 ^{c}	82.9 ^{c}	81.9 ^{c}

Those parameters were taken 21 days after exposure to the corresponding treatment. All the plants were transferred to *ex vitro* conditions and after 42 days, the survival (%) was evaluated. Ventilation: without filter (-) and with filter (+). Data are mean ± SD of three independent experiments, each with three replicates ($n = 6$). Treatments with the same letters are not significantly different (ANOVA followed by Least Significance Different (LSD) Duncan`s post-test; * $p<0.05$) was used.

3.4.2. Genome-wide identification of CpAux/IAA and CpARFs involved in rhizogenesis.

A total of 18 Aux/IAA sequences were found (Table 3.2) and 12 ARFs sequences (Table 3.3) identified in *C. papaya* from in Plant Transcription Factor database (PlnTFDB 3.0). All the sequences showed high percentages of identity (77 to 90 %) and high percentages of similarity (82 to 95 %) with expected E values <7E-11 to <1E-180 in comparison with *A. thaliana*. In relation to the pair-wise comparison analysis of the predicted protein sequences of Aux/IAA family, CpAux/IAA12 showed high percentage of identity with AtAux/IAA12 (72.9 %), CpAux/IAA14 showed a high percentage identity with AtAux/IAA14 (72.9 %) and finally CpAux/IAA13 with AtAux/IAA28 (72.9 %) (Table 3.4). In ARFs family, CpARF5 showed greater identity with AtARF5 (89.8 %), CpARF6 showed high identity with AtARF6 (96.7 %) while that CpARF7 showed high identity with AtARF7 and AtARF19 (92.1 % for both sequences) (Table 3.5).

Table 3.2. BLASTP results of the genomic sequences CpAux/IAA genes found in the *C. papaya* cv. Sun Up genome, that show homology to Aux/IAA genes of *A. thaliana.*

Number sequences	Query Accnum	Segment lenght (bp)	% Identity	% Similarity	E value
	AUX/IAA				
1	**supercontig_10.173**	354	89	89	1E-178
2	**supercontig_12.32**	453	96	97	1E-189
3	**supercontig_129.23**	342	88	92	1E-111
4	**supercontig_1346.4**	324	89	93	1E-132
5	**supercontig_1476.1**	36	89	94	2E-100
6	**supercontig_217.3**	457	91	97	1E-104
7	**supercontig_2282.1**	476	92	95	1E-156
8	**supercontig_23.159**	355	95	96	1E-189
9	**supercontig_23.62**	445	89	88	1E-129
10	**supercontig_52.93**	321	86	91	1E-111
11	**supercontig_52.94**	296	90	90	1E-129
12	**supercontig_57.25**	352	78	88	1E-167
13	**supercontig_58.36**	344	79	90	1E-198
14	**supercontig_58.37**	298	85	89	1E-99
15	**supercontig_59.6**	293	89	94	1E-129
16	**supercontig_65.69**	343	95	97	1E-182
17	**supercontig_87.29**	369	90	91	1E-176
18	**supercontig_946.4**	333	88	89	1E-102

Table 3.3. BLASTP results of the genomic sequences CpARFs genes found in the *C. papaya* cv. Sun Up genome, that show homology to ARFs genes of *A. thaliana*

Number sequences	Query Accnum	Segment lenght (bp)	% Identity	% Similarity	E value
1	supercontig_7.3	1349	82	87	1E-103
2	supercontig_9.161	1101	84	89	1E-174
3	supercontig_17.53	1762	90	90	1E-121
4	supercontig_26.24	1829	85	89	1E-130
5	supercontig_49.122	1521	84	91	2E-109
6	supercontig_53.88	1299	82	86	1E-101
7	supercontig_65.4	1342	88	92	1E-134
8	supercontig_96.40	1645	89	93	1E-109
9	supercontig_139.80	1023	77	82	1E-123
10	supercontig_173.22	842	85	91	1E-178
11	supercontig_261.2	1559	88	95	1E-177
12	contig_31756.1	1243	80	88	1E-138

Table 3.4. Pairwise percentage identity of 18 aminoacids sequences Aux/IAA identified in *C. papaya* and their 29 orthologs aminoacids sequences identified in *A. thaliana*, the highest percentage is enclosed in bold and italics.

		Aux/IAA 1	Aux/IAA 2	Aux/IAA 3	Aux/IAA 4	Aux/IAA 5	Aux/IAA 6	Aux/IAA 7	Aux/IAA 8	Aux/IAA 9	Aux/IAA 10	Aux/IAA 11	Aux/IAA 12	Aux/IAA 13	Aux/IAA 14	Aux/IAA 15	Aux/IAA 16	Aux/IAA 17	Aux/IAA 18	Aux/IAA 19	Aux/IAA 20	Aux/IAA 26	Aux/IAA 27	Aux/IAA 28	Aux/IAA 29	Aux/IAA 30	Aux/IAA 31	Aux/IAA 32	Aux/IAA 33	Aux/IAA 34
1	**supercontig_10.158**	42.4	42.4	42.4	42.4	30.6	30.6	53.0	53.0	53.0	30.6	17.3	53.0	53.0	53.0	42.4	62.5	53.0	30.6	30.6	-16.3	53.0	42.4	17.3	1.9	-16.3	1.91	-16.3	-38.6	30.6
2	**supercontig_12.34**	62.5	62.5	62.5	71.2	42.4	62.5	62.5	71.2	53.0	30.6	30.6	42.4	42.4	62.5	30.6	62.5	62.5	42.4	53.0	17.3	62.5	62.5	30.6	1.9	17.3	-16.3	-16.3	-67.3	42.4
3	**supercontig_129.23**	62.5	53.0	62.5	62.5	30.6	42.4	53.0	71.2	62.5	17.3	42.4	42.4	42.4	53.0	42.4	71.2	53.0	30.6	53.0	30.6	53.0	53.0	30.6	-16.3	17.3	1.9	-16.3	-1.9	30.6
4	**supercontig_1346.4**	71.2	71.2	71.2	71.2	42.4	62.5	53.0	62.5	62.5	17.3	30.6	30.6	30.6	62.5	30.6	62.5	53.0	42.4	53.0	30.6	62.5	53.0	***79.2***	1.9	30.6	-16.3	-16.3	-1.9	42.4
5	**supercontig_1476.1**	71.2	62.5	71.2	71.2	53.0	53.0	79.2	79.2	53.0	42.4	42.4	42.4	42.4	62.5	30.6	86.6	71.2	53.0	53.0	17.3	62.5	62.5	53.0	17.3	17.3	1.9	1.9	-38.6	53.0
6	**supercontig_217.3**	-38.6	-38.6	-38.6	-38.6	-16.3	1.9	1.9	1.9	-38.6	1.9	-16.3	1.9	1.9	-16.3	-38.6	1.9	-16.3	1.9	-38.6	-16.3	-16.3	1.9	30.6	42.4	-16.3	1.9	-38.6	-16.3	1.9
7	**supercontig_2282.1**	62.5	53.0	62.5	62.5	42.4	42.4	62.5	86.6	71.2	30.6	30.6	30.6	30.6	62.5	30.6	79.6	53.0	30.6	53.0	17.3	53.0	53.0	53.0	1.9	17.3	1.9	-16.3	-67.3	30.6
8	**supercontig_23.140**	17.3	30.6	17.3	17.3	42.4	42.4	42.4	42.4	30.6	17.3	30.6	17.3	17.3	30.6	42.4	30.6	30.6	1.9	30.6	-16.3	17.3	30.6	17.3	-16.3	-16.3	-16.3	1.9	1.9	1.9
9	**supercontig_23.52**	-16.3	-16.3	-16.3	1.9	1.9	17.3	-38.6	-16.3	-38.6	1.9	-67.3	-38.6	-38.6	-38.6	-16.3	-16.3	-38.6	-67.3	-16.3	-38.6	-38.6	-16.3	-38.6	-1.2	-38.6	-67.3	-67.3	-1.9	-38.6
10	**supercontig_52.88**	62.5	53.0	62.5	62.5	42.4	42.4	62.5	71.2	53.0	17.3	30.6	30.6	30.6	53.0	30.6	79.2	53.0	30.6	62.5	17.3	53.0	53.0	42.4	-16.3	17.3	-16.3	-16.3	-67.3	30.6
11	**supercontig_57.24**	-16.3	-16.3	17.3	-16.3	-38.6	-16.3	-16.3	-16.3	-16.3	1.9	-38.6	-38.6	-38.6	-16.3	1.9	-16.5	-16.3	-38.6	1.91	53.0	-38.6	17.3	-16.3	1.9	42.4	30.6	-38.6	-38.6	1.9
12	**supercontig_58.26**	79.2	62.5	86.6	79.2	42.4	42.4	62.5	62.5	53.0	30.6	42.4	30.6	30.6	62.5	30.6	71.2	62.5	30.6	71.2	30.6	53.0	62.5	30.6	-16.3	30.6	1.9	17.3	-1.4	30.6
13	**supercontig_58.29**	62.5	62.5	53.0	53.0	42.4	42.4	71.2	71.2	62.5	17.3	30.6	30.6	30.6	***79.2***	30.6	71.2	71.2	30.6	53.0	1.9	42.4	42.4	30.6	-16.3	1.9	-16.3	1.9	-38.6	30.6
14	**supercontig_59.8**	1.9	1.9	1.9	1.9	-16.3	1.9	17.3	30.6	17.3	17.3	53.0	***79.2***	71.2	17.3	-16.3	1.9	17.3	30.6	-16.3	-67.3	42.4	1.9	1.9	1.91	-67.3	-16.3	-16.3	-1.7	30.6
15	**supercontig_65.68**	17.3	17.3	17.3	17.3	-16.3	1.9	1.9	1.9	1.9	17.3	17.3	17.3	17.3	1.9	-16.3	1.9	1.9	17.3	17.3	17.3	17.3	-16.3	1.9	-16.3	17.3	17.3	53.0	-67.3	-1.7
16	**supercontig_87.24**	30.6	30.6	30.6	30.6	17.3	17.3	30.6	30.6	30.6	30.6	86.6	79.2	79.2	30.6	30.6	42.4	30.6	42.4	17.3	-16.3	62.5	17.3	30.6	17.3	-16.3	1.9	1.9	-1.9	42.4
17	**supercontig_946.4**	62.5	42.4	62.5	53.0	42.4	53.0	42.4	53.0	42.4	1.9	17.3	30.6	30.6	42.4	30.6	62.5	42.4	17.3	79.2	30.6	53.0	42.4	17.3	-16.3	42.4	1.9	1.9	-1.9	-1.7
18	**supercontig_52.89**	62.5	62.5	62.5	71.2	62.5	53.0	62.5	62.5	42.4	30.6	30.6	30.6	30.6	53.0	30.6	62.5	62.1	30.6	42.4	1.9	53.0	62.5	17.3	-16.3	1.91	-38.6	1.9	-67.3	30.6

Table 3.5. Pairwise percentage identity of 12 aminoacids sequences ARFs identified in *C. papaya* and their 23 orthologs aminoacids sequences identified in *A. thaliana*, the highest percentage is enclosed in bold and italics.

		ARF 1	ARF 2	ARF 3	ARF 4	ARF 5	ARF 6	ARF 7	ARF 8	ARF 9	ARF 10	ARF 11	ARF 12	ARF 13	ARF 14	ARF 15	ARF 16	ARF 17	ARF 18	ARF 19	ARF 20	ARF 21	ARF 22	ARF 23
1	**supercontig_7.3**	39,9	47,4	***79,8***	56,1	35,9	41,8	41,8	41,8	33,8	33,8	41,8	25,1	25,1	22,8	22,8	25,1	15,6	41,8	39,9	20,5	20,5	22,8	15,6
2	**supercontig_9.161**	56,1	61,0	39,9	45,6	33,8	39,9	41,8	41,8	52,7	41,8	59,4	27,4	27,4	29,6	29,6	29,6	13,0	56,1	41,8	22,8	22,8	27,4	20,5
3	**supercontig_17.53**	47,4	45,6	41,8	47,4	65,7	***96,7***	77,1	89,8	39,9	33,8	49,2	20,5	20,5	13,0	15,6	27,4	22,8	47,4	77,1	13,0	13,0	13,0	5,0
4	**supercontig_26.24**	43,7	41,8	39,9	45,6	***89,8***	67,2	67,2	65,7	43,7	22,8	39,9	22,8	22,8	15,6	18,0	15,6	22,8	33,8	70,1	15,6	18,0	15,6	7,8
5	**supercontig_49.122**	15,6	7,83	25,1	18,0	10,4	22,8	18,0	18,0	20,5	35,9	20,5	-9,8	-9,8	-6,6	-3,6	25,1	69,4	18,0	15,6	-6,6	-6,6	-9,8	13,1
6	**supercontig_53.88**	31,7	27,4	31,5	25,1	20,5	31,7	25,1	31,7	27,4	68,6	35,9	13,0	13,0	15,6	18,0	71,5	31,7	33,8	31,7	10,4	10,4	13,0	5,0
7	**supercontig_65.4**	33,8	33,8	33,8	27,4	27,4	33,8	29,6	35,9	33,8	75,7	37,9	10,4	10,4	13,0	15,6	65,7	35,9	35,9	39,9	10,4	13,0	10,4	2,2
8	**supercontig_96.40**	74,4	61,0	37,9	43,7	39,9	43,7	43,7	45,6	70,1	39,9	79,8	35,9	35,9	39,9	39,9	25,1	27,4	74,4	43,7	35,9	35,9	39,9	31,7
9	**supercontig_139.80**	43,7	41,8	54,4	88,6	37,9	45,6	47,4	45,6	39,9	33,8	43,7	20,5	20,5	18,0	18,0	18,0	5,09	43,7	43,7	15,6	15,6	18,0	10,4
10	**supercontig_173.22**	79,0	45,6	10,4	27,4	18,0	25,1	29,6	29,6	43,7	15,6	51,0	27,4	27,4	15,6	13,0	-0,6	-9,8	47,4	25,1	10,4	10,4	13,0	13,0
11	**supercontig_261.2**	51,0	49,2	43,7	47,4	70,1	79,8	***92,1***	79,8	39,9	31,7	47,4	29,6	29,6	20,5	22,8	22,8	22,8	43,7	***92,1***	20,5	20,5	20,5	13,0
12	**contig_31756.1**	70,1	84,9	39,9	45,6	35,9	39,9	47,4	41,8	57,8	33,8	64,1	29,6	29,6	35,9	33,8	22,8	22,8	62,6	43,7	29,6	29,6	33,8	25,1

3.4.3. Alignment and phylogenetic analysis of CpAux/IAA and CpARFs proteins

With respect to Aux/IAA, the alignment analysis of the aminoacids sequences of AtAux/IAA12, AtAux/IAA14 and AtAux/IAA28 with their orthologs CpAux/IAA12, CpAux/IAA13 and CpAux/IAA14 (recognized by being auxin transcription repressors involved in root formation) showed 4 characteristics domains (I - IV domain), In the Figure 3.1, I domain is homodimer, II domain showed the motifs of aminoacids (QVVGWPPVRSYRK) and finally was observed the homo- and heterodimerization domain (III and IV domain) which interact with the domains III and IV of ARFs family in absence of auxin. The phylogenetic tree from Aux/IAA predicted proteins showed five subclades, of which the sequences CpAux/IAA12 and CpAux/IAA13 are in subclade IV, only CpAux/IAA14 is grouped in the subclade II (Figure 3.2). Based on the phylogenetic tree, three sister pairs were identified between *A. thaliana* and *C. papaya*: CpAux/IAA12 with AtAux/IAA12, CpAux/IAA13 with AtAux/IAA13 and CpAux/IAA14 with AtAux/IAA14.

In the Figure 3.3, the alignment analysis of the aminoacids sequences of AtARF5, AtARF6, AtARF7, AtARF8 and AtARF19 with their CpARF5, CpARF6 and CpARF7 orthologs sequences (recognized by being auxin transcription involved in root formation) is presented. Family members showed in their aminoacids sequences an amino-terminal DNA binding domain (DBD), a medium region enriched with serine, proline, leucine and glycine residues, we also observed at the III and IV domains of homo- and heterodimerization located in the carboxyl-terminal dimerization domain (CTD). The phylogenetic analysis of ARFs (Figure 3.4) family showed that the predicted proteins are grouped in six subclades, however the AtARF5, AtARF6, AtARF7, AtARF8 and AtARF19 sequences of *A. thaliana* and the CpARF5, CpARF6 and CpARF7 sequences of *C. papaya* were all grouped in the subclade II. Based on the phylogenetic analysis, CpARF5 was groped with AtARF5, CpARF6 was grouped with AtARF6, and finally CpARF7 was grouped with AtARF7 and AtARF19 (Figure 3.4).

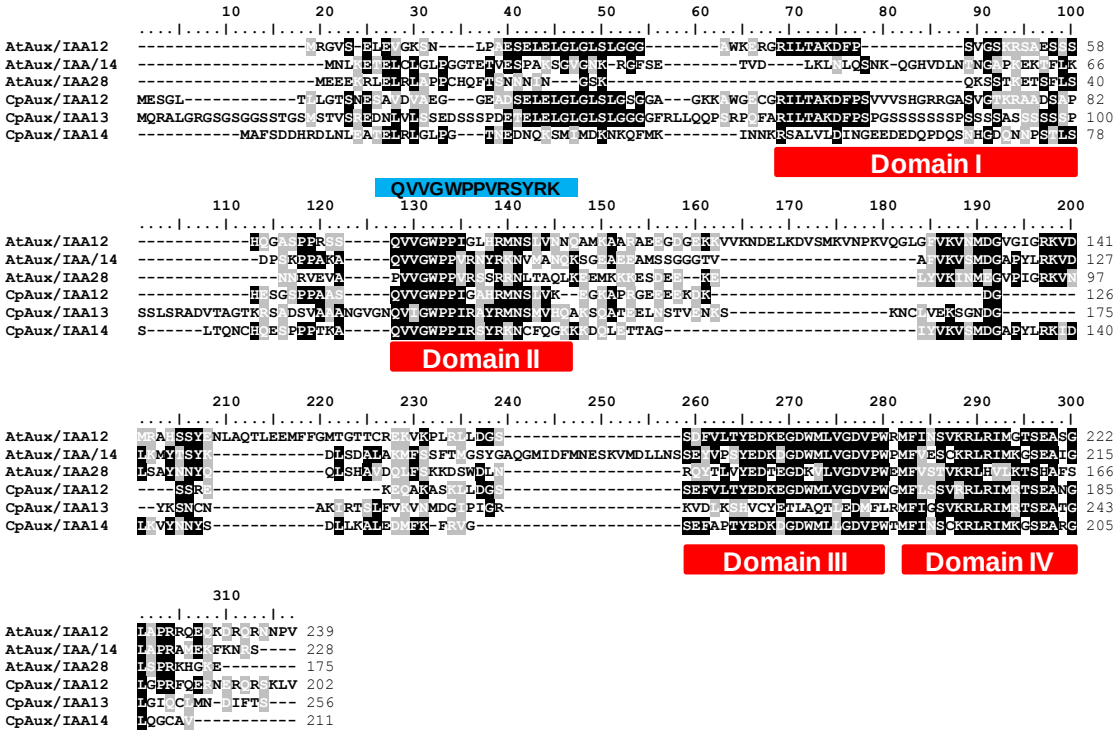

Figure 3.1. ClustalX alignment of the deduced amino acid sequences of *C. papaya* (CpAux/IAA12, CpAux/IAA13 and CpAux/IAA14) and *A. thaliana* (AtAux/IAA12, AtAux/IAA14 and AtAux/IAA28) Aux/IAA orthologs. Identical amino acids are shaded in black and conservative substitutions are shaded in grey while the other amino acids are different. The bars of colors predicted the characteristics domains of Aux/IAA family, in red box are showed the domain I, II, III and IV, in blue box are showed the amino acids QVVGWPPVRSYRK of II domain.

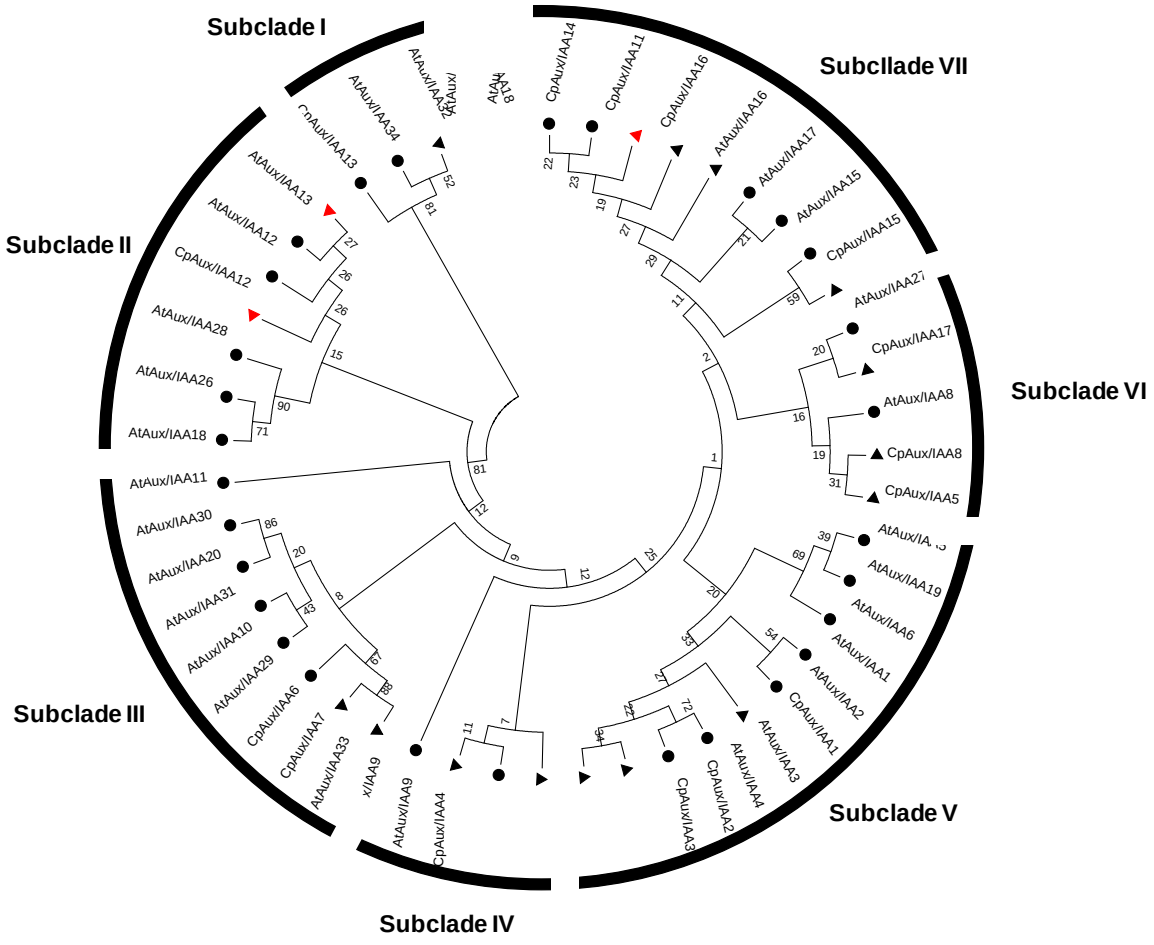

Figure 3.2. Neighbor-joining phylogenetic tree of Aux/IAA aminoacids sequences from *A. thaliana* and *C. papaya* cv. Sun Up showing the formed 7 subclades. Aux/IAA orthologs sequences from *A. thaliana* are shown in black circles and Aux/IAA orthologs of *C. papaya* in triangles, only the CpAux/IAA involved in inhibiting rooting are shown in red triangles (CpAux/IAA12, CpAux/IAA13 and CpAux/IAA14).

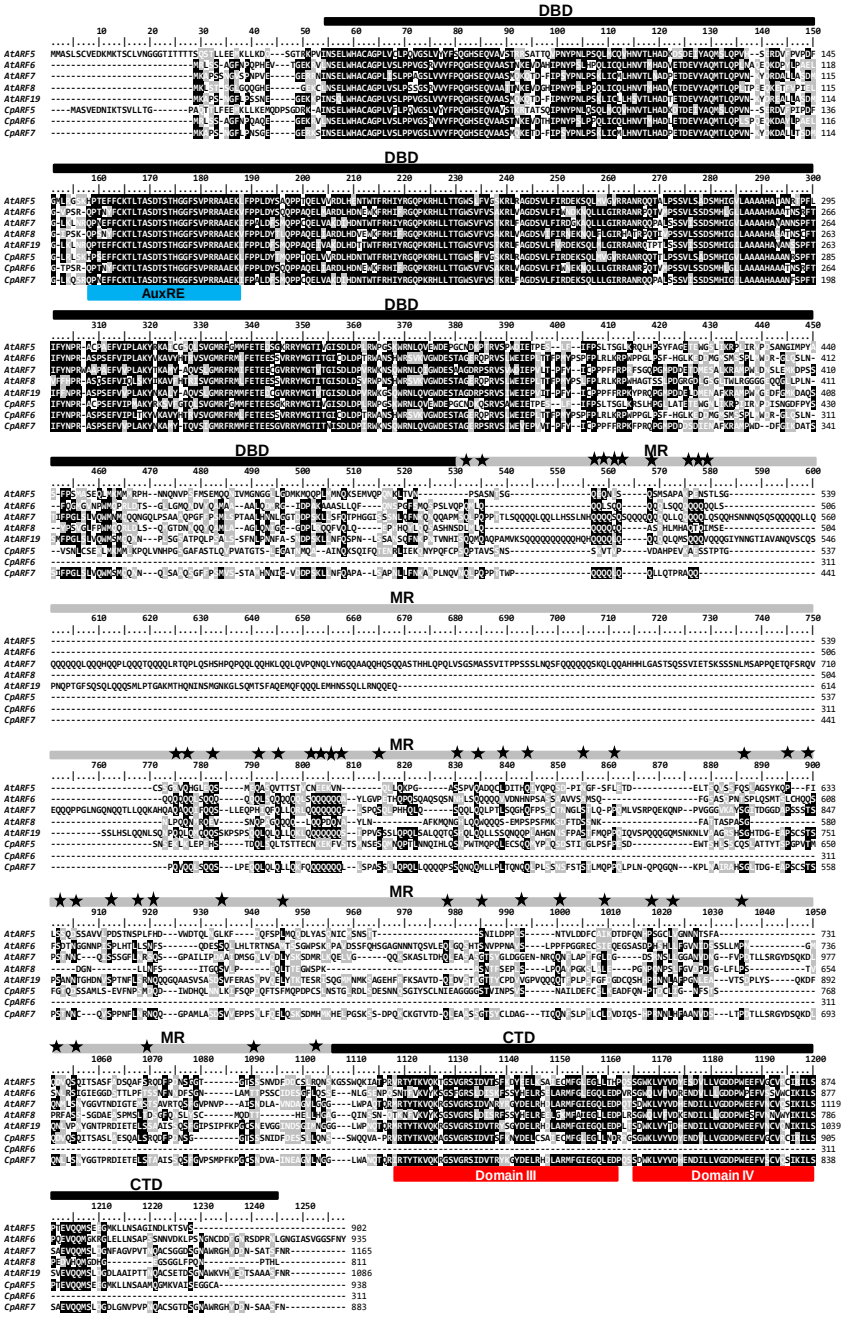

Figure 3.3. ClustalX alignment of the deduced amino acid sequences of *C. papaya* (CpARF5, CpARF6 and CpARF7 and A. *thaliana* (AtARF5, AtARF6, AtARF7, AtARF8 and AtARF19) ARF orthologs. Identical amino acids are shaded in black and conservative substitutions are shaded in grey while the other amino acids are different. Multiple alignments of the DBD and CTD regions are shown in black line; the MR region is shown in gray line. In blue box is observed the site auxin response element (ARE) in the domain DBD, the black stars showed thein middle region rich in glycine (Q), leucine (L), serine (S). In red box the III and IV domains in CTD.

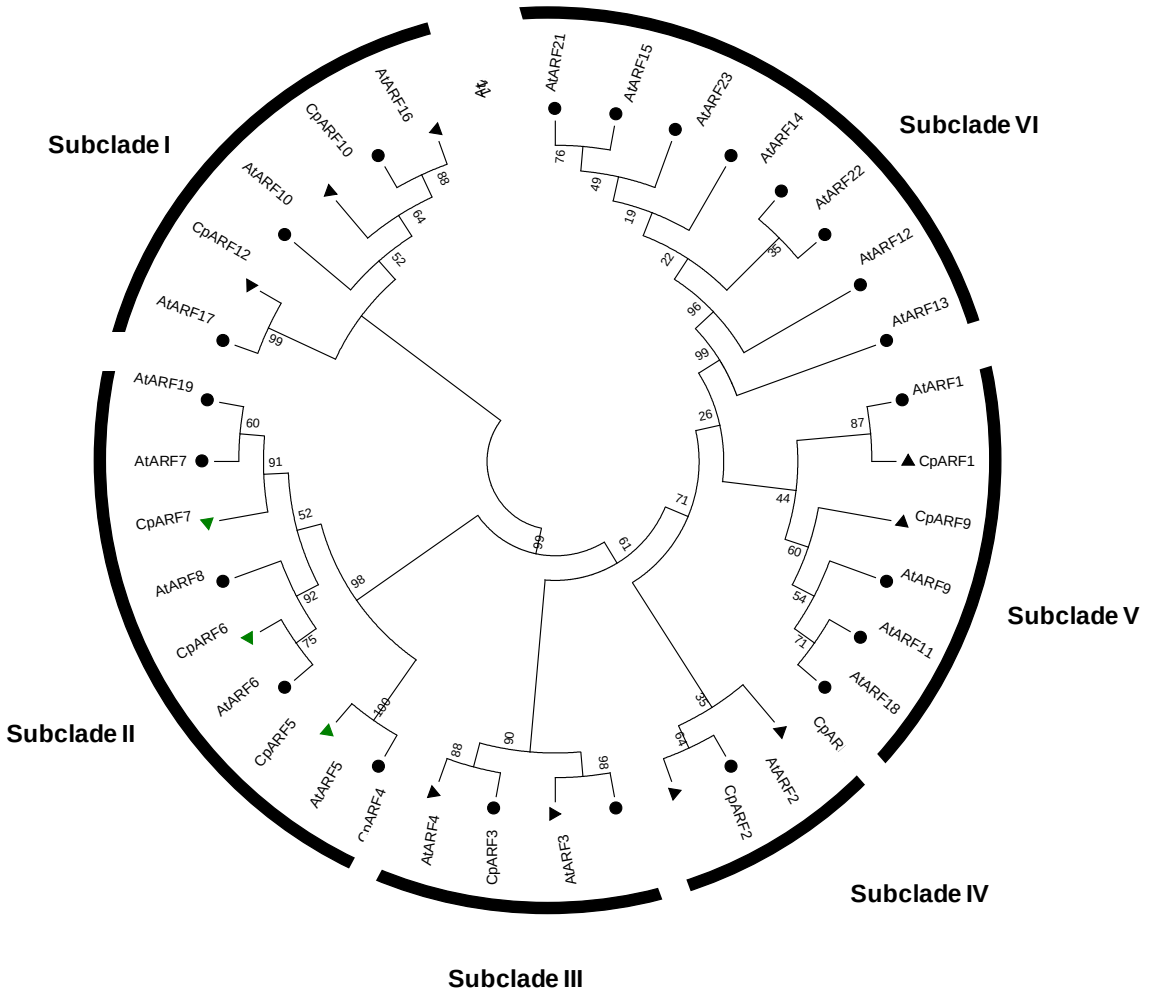

Figure 3.4. Neighbor-joining phylogenetic tree of ARFs aminoacids sequences from *A. thaliana* and *C. papaya* cv. Sun Up showing the formed 6 subclades. ARFs orthologs sequences from *A. thaliana* are shown in black circles and ARF orthologs of *C. papaya* in triangles, only the CpARFs involved on rooting are shown in green triangles (CpARF5, CpARF6 and CpARF7).

3.4.4. Expression of CpAux/IAA genes in rhizogenic treatments

In Figure 3.5 a-b we present the relative expression levels (REL) in SB and R tissues of the experiment on sucrose concentrations. Seedlings from S1 and S2 treatments showed low REL values in SB and R tissues of CpAux/IAA12, CpAux/IAA13 and CpAux/IAA14 genes, while de-rooted seedlings showed REL high values (1.8 to 2.8 in SB tissue and values of 1.2 until 1.8 in R tissue). *In vitro* plantlets from S5 and S6 treatments (plantlets that did not generate roots) showed the highest REL of CpAux/IAA13 gene in SB tissue (Figure 3.5 a). On the contrary, *in vitro* plantlets that did generate roots (S7 and S8 treatment) showed intermediate REL values (3.5 to 4.8) of those genes in SB tissue. In R tissue, *in vitro* plantlets with or without sucrose (S7 and S8 treatments) supplemented with exogenous IBA showed high REL values of these repressor genes (Figure 3.5 b).

In the light experiment (Figure 3.5 c-d) the seedlings showed lower expression in SB tissue when they were exposed to low light intensity, however, exposed to high light intensity the REL values were even lower. De-rooted seedlings with low light intensity and exogenous IBA showed high REL values of CpAux/IAA12, CpAux/IAA13 and CpAux/IAA14 in SB tissue. On the contrary, when they were exposed to high light intensity the REL values of these genes went down (Figure 3.5 c). Interestingly, *in vitro* plantlets were able to form adventitious roots when exposed to high light intensity even without the addition of exogenous IBA, but their REL values in SB and R tissues. *In vitro* plantlets from L8 treatment showed similar REL values of CpAux/IAA12-13-14 genes in SB and R tissues when compared with de-rooted seedlings from L3 treatment (treated with exogenous IBA but with low light intensity).

As shown in Figure 3.5 e-f; seedlings showed a low expression of the repressor genes in both SB and R tissues, de-rooted seedlings without ventilation showed lower REL of CpAux/IAA12-13-14 genes in SB and R tissues. However when these de-rooted seedlings were ventilated their REL values decreased. Interestingly, non-ventilated *in vitro* plantlets without IBA showed high values REL for all repressor genes in SB tissue. Non-ventilated *in vitro* plantletsbut with exogenous IBA showed high REL values of these genes in SB and R tissues. While in ventilated *in vitro* plantlets with exogenous IBA showed low REL values of CpAux/IAA12-13-14 genes (Figure 3.5 e, f).

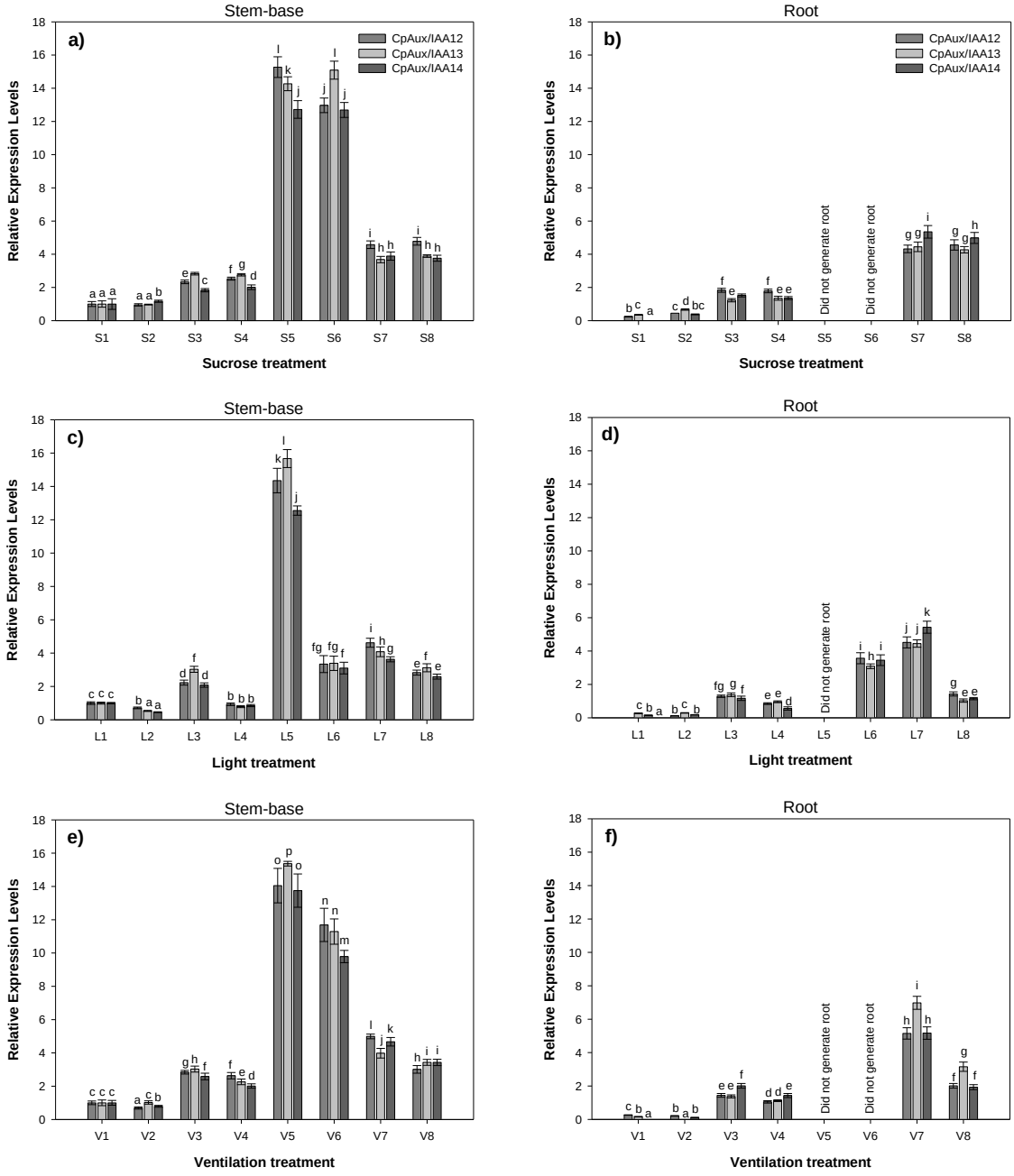

Figure 3.5. qRT-PCR analysis of CpAux/IAA12, CpAux/IAA13 and CpAux/IAA14 genes expression in SB (**a**, **c**, **e**) and R (**b**, **d**, **f**) tissues of seedlings, de-rooted seedlings and *in vitro* plantlets of *C. papaya* cv. Maradol exposed during 21 days in three treatments different named: Sucrose (**a**, **b**), light (**c**, **d**) and Ventilation (**e**, **f**). The relative mRNA level of individual genes was normalized with respect to that of CpEF1a gene. The data are mean ± SD from three independent experiments each with three replicates (*n = 3*). Different letters indicate significant differences among means ($p < 0.05$).

3.4.5. Expression of CpARFs genes in rhizogenic treatments

The expression analysis revealed that in the sucrose experiment (Figure 3.6 a-b) the seedlings as well as de-rooted seedlings showed higher REL values of the CpARF5, CpARF6 and CpARF7 genes in R tissue. In the treatments that did not generate roots (S5 and S6), obviously the expression of these genes were not performed in root tissues, however these *in vitro* plantlets showed the lowest REL values of CpARF5, CpARF6 and CpARF7 genes in SB tissue (Figure 3.6 a). Furthermore, when these *in vitro* plantlets were exposed to exogenous IBA the REL values increased for both tissues. CpARF7 gen for S7 and S8 treatment showed higher REL values (4.5 and 5.3 respectively) in comparison to CpARF5 and CpARF6 REL values in root tissue (Figure 3.6 b).

In light experiment, it was shown that seedlings, de-rooted seedlings and *in vitro* plantlets when are exposed to high light intensity the REL values of the CpARF5, CpARF6 and CpARF7 genes increased in both SB and R tissues (Figure 3.6 c-d). *In vitro* plantlets of L5 treatment that did not generate roots, showed a very low expression of the CpARF5, CpARF6 and CpARF7 genes in SB tissue (Figure 3.6 c). Interestingly, *in vitro* plantlets from L6 treatment even without the addition of exogenous IBA, the *in vitro* plantlets were able to generate roots when their light intensity was increased to 750 µmol photon m^{-2} s^{-1}, this was the only treatment non treated with IBA in the culture medium, that showed intermediate REL values of CpARF5, CpARF6 and CpARF7 genes in both SB and R tissues (Figure 3.6 d).

In the ventilation experiment (Figure 3.6 e-f), when the seedlings had ventilation the REL values increased for the CpARF5, CpARF6 and CpARF7 genes, however in de-rooted seedlings grown with ventilation, those REL values were lower in SB tissue (Figure 3.6 e). *In vitro* plantlets with or without ventilation but without exogenous IBA, showed low REL values in both SB and R tissues, but when IBA was added to the culture medium, to plantlets grown either with or without ventilation, they showed high REL of CpARF5, CpARF6 and CpARF7 genes (with REL values from 3 to 4) for SB tissues (Figure 3.6 e) and of 3 to 8.5 in R tissues (Figure 3.6 f). *In vitro* plantlets treated with IBA and growing with ventilated vessels, the CpARF5 and CpARF7 genes showed higher REL in comparison to CpARF6 with lower REL values.

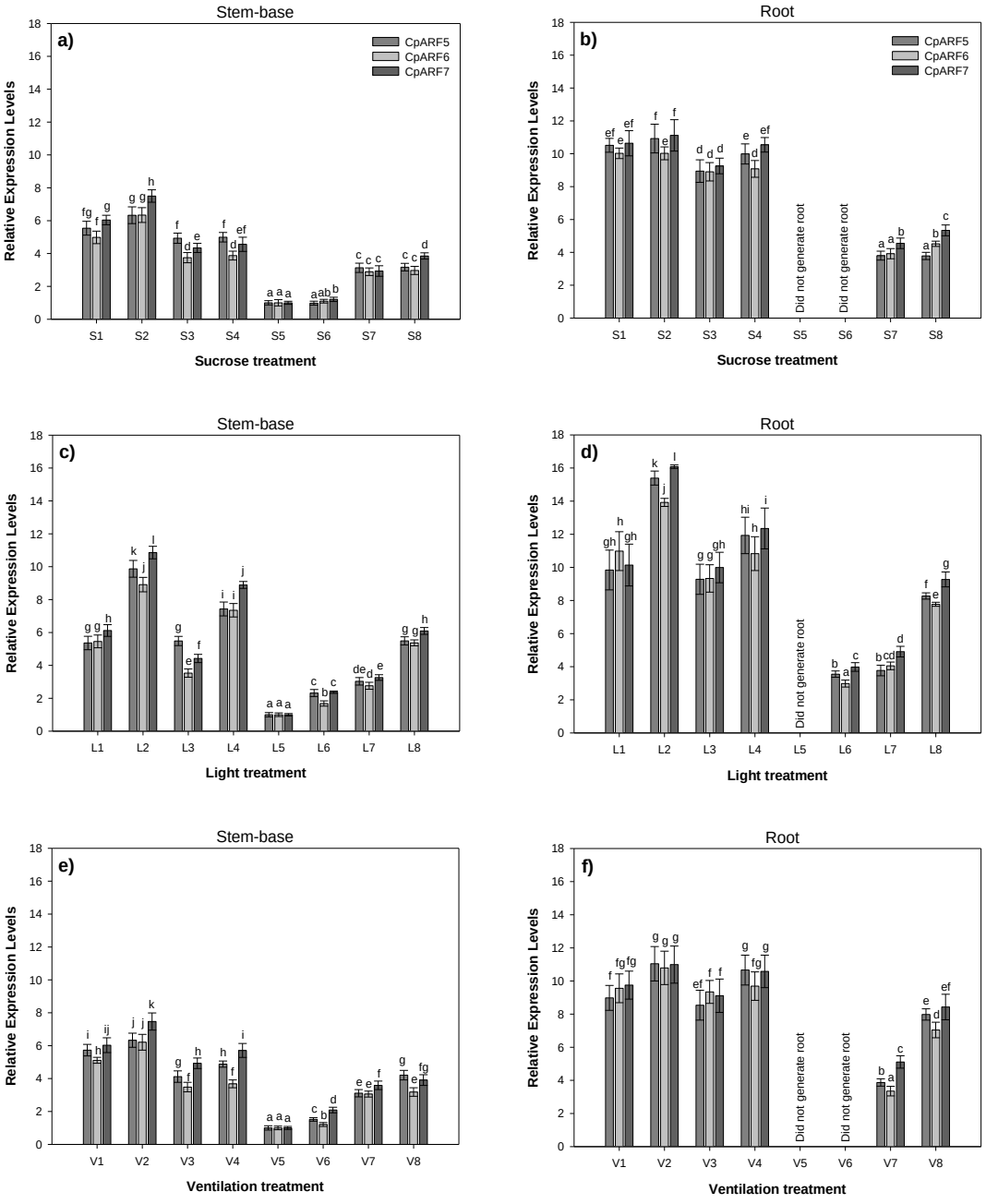

Figure 3.6. qRT-PCR analysis of CpARF5, CpRF6 and CpARF7 genes expression in SB (**a**, **c**, **e**) and R (**b**, **d**, **f**) tissues of seedlings, de-rooted seedlings and *in vitro* plantlets of *C. papaya* cv. Maradol exposed during 21 days in three treatments different named: Sucrose (**a**, **b**), light (**c**, **d**) and Ventilation (**e**, **f**). The relative mRNA level of individual genes was normalized with respect to that of CpEF1a gene. The data are mean ± SD from three independent experiments each with three replicates ($n = 3$). Different letters indicate significant differences among means ($p < 0.05$).

3.5. DISCUSSION

The *in vitro* environment might affect plant performance when transferred *ex vitro,* conditions such as high relative humidity levels, constant temperatures, low photosynthetic photon flux density (PPFD), large diurnal fluctuations in CO_2 concentration, high concentrations of sugar, salts and growth regulating substances in the medium they should be considered (Zobayed et al., 2004; Xiao et al., 2011). *C. papaya* plantlets grown under these *in vitro* conditions, often lead to poor development of cuticule, low rates of transpiration, low photosynthesis, low uptake of water and nutrient, and a high rate of dark respiration, all of which result in poor growth *in vitro* and, subsequently, decrease adaptability during the acclimatization period. Another problem that presents this species is its difficulty to develop a good root system. It is necessary first to grow the shoots, and then use special methods to induce rooting in a so-called rooting stage (Yu et al., 2000). Therefore, *in vitro* rooting is essential for any micropropagation protocol of *C. papaya* because it will result in a successful acclimatization of the plants obtained. To counteract this problem in *C. papaya,* 2 mg L^{-1} of Indole-3-butyric acid (IBA) auxin has been used in order to increase rooting percentage, and root number produced by *in vitro* plantlet (Drew, 1987; Drew et al., 1993).

Thus, changing sucrose concentrations (Table 3.1), it was observed that without the addition of IBA, the *in vitro* plantlets did not generate roots, however, when the *in vitro* plantlets were exposed to 20 g L^{-1} of sucrose and IBA (S8 treatment) plants were able to generate roots, but they showed low survival at 42 days under *ex vitro* conditions. Besides, the concentration of sucrose added to the *in vitro* plantlets did not influence their rooting or survival rates (S7 and S8 treatments). Respect to the interaction between auxin (exogenous) and glucose (sucrose), in peony shoots regulated the roots formation, also is was stimulate shoot growth or rooting and was inhibit renewal bud formation. (Gabryszewska, 2010). In terms of root growth, lateral root induction, root hair elongation and gravitropism, glucose may use auxin signal transduction elements to affect root architecture (Mishra et al., 2009), however, is possible that 20 mg L^{-1} of sucrose used in our experiment, no excessive accumulation occurs of hexoses and starch that affect feedback inhibition of photosynthesis of the *in vitro* plantlet, as mentioned by Le et al. (2001). With respect to the changes of expression of the auxin transcriptional genes in

sucrose experiment, several studies have shown that glucose (sucrose) can affect almost all steps of auxin metabolism since it can modulate auxin biosynthetic gene YUCCA2, auxin receptors TIR1 and ABP1, auxin transporter PIN1, auxin response factors ARF4, ARF8 and a number of genes belonging to auxin induced gene families such as AUX/IAA (Mishra et al., 2009). However, our results suggest that even concentrations of 20 mg L^{-1} of sucrose do not affect auxin-regulated gene expression; also, this concentration was not able to induce a greater number of roots on *in vitro* plantlets of *C. papaya*.

Another feature that we consider important to change in order to improve the survival of plants obtained from *in vitro* conditions was the light intensity supplied to the *in vitro* plantlets before being transferred to *ex vitro* conditions; this is because those *in vitro* plantlets have a substantial reduction in their photosynthesis that occurs by heterotrophic conditions of *in vitro* culture, this alters photosystems (or at least of PSII) and it probably has not been result of any major effects on other aspects of photosynthesis (Dewir et al., 2005). Therefore, our think that an increases of the photosynthetic capacity (in this case, high PPFD) of *in vitro* plantlets may further improve plant establishment under *ex vitro* conditions, as suggested by (Kozai and Iwanami, 1988; Kitaya et al., 1995; Hahn and Paek, 2001). Interestingly, *in vitro* plantlets exposed to high light intensity (750 µmol photon m^{-2} s^{-1}) and without IBA in their culture medium (L6 treatment), were the only *in vitro* plantlets of the three experiments performed (Sucrose, Light and Ventilation) capable of forming roots without the presence of IBA. In *A. thaliana*, light triggers auxin synthesis in developing young leaves (Bhalerao et al., 2002), the auxin endogenous generated is distributed through the seedling (Grieneisen et al., 2007) where is transported to individual cells and they provide a mechanism for light to drive both tissue-specific responses and coordinate development between the shoot and root, in specific the lateral root emergence and primary root elongation (Halliday and Fankhauser, 2003; Salisbury et al., 2007; Halliday et al., 2009). The best results were obtained when those *in vitro* plantlets were supplemented with IBA in their culture medium (L8 treatment), thereby showed high survival rate (87 %). Much has been reported of the effects of that the light imposes a strong influence on multiple facets of the auxin system, controlling auxin levels, transport, and responsiveness. In this context, Sorín et al. (2005) they mentioned that AGO1 gen through of the action of ARF17 expression, regulates genes involved at the cross talk between auxin and light signaling during adventitious root development in *A. thaliana*. In

our study, we observed that high light intensity increasing the expression of CpARF5, CpARF5, and CpARF7 genes in roots was found to be upregulated by high light intensity. Therefore, our think that this crosstalk light-auxin provid a mechanism for light to drive both tissue-specific responses and roots formation.

Under natural *in vitro* conditions, the CO_2 concentration inside the culture vessels increase during the dark periods and decreases during the light periods because of *in vitro* plantlets respiration and photosynthesis, these conditions in the vessels on *in vitro* culture, affects to *in vitro* plantlets during their growth and survival *ex vitro* acclimatization (Solárová and Pospíšilová, 1997; Hahn and Paek, 2001; Xiao et al., 2011). In our study we think that the non-ventilated *in vitro* plántlets generate a higher content of ethylene causing inhibition of adventitious roots, in addition low CO_2 content of the *in vitro* environmnet affects even more its photosynthetic rate. Likewise, Khalafalla and Hattori (2000), have reported that high concentrations of ethylene may influence the inhibition of adventitious roots under *in vitro* conditions in *Vicia faba* L., however when they supplemented ethylene inhibitors (AgNO3), improved the emergence of the roots and increased the number of roots per shoot, root growth rate and root length. Besides, Růžička et al., (2007) they reported that althought the ethylene does not affect cell cycle activity nor the size of the root meristems, if it strongly reduces the elongation of epidermal cells, therefore the ethylene inhibits root formation (Ivanchenko et al., 2008; Negi et al., 2008; Negi et al., 2010). Therefore, our utilized ventilated systems with the objective of remove growth gases as ethylene and to increase the CO_2 environmental. Thus, *in vitro* plantlets were rooted in culture flasks with plastic lid pierced with a diameter of 1 cm^2 (with filter), under this condition the *in vitro* plantlets with filter and supplemented with IBA in culture medium (V8 treatment) to form roots, with this ventilation system improved the quality of the *in vitro* plantlets and their survival rate (79.7 %). This crosstalk ethylene-auxin to root formation modulates biosynthetic genes. For example, ethylene has been shown to promote auxin transport from the meristem towards the root elongation zone, where the resulting auxin increase triggers the well-known ethylene-mediated root growth inhibition (Lewis et al., 2011).

Increased auxin concentration through exogenous application or genetic manipulation has shown good results in the formation of lateral roots (Malamy, 2005), at the molecular level, auxin rapidly activates the transcription levels of numerous genes (Abel and Theologis,

1996). Thus, auxin effects, including lateral root formation, may be mediated directly through changes in gene expression of auxin regulatory genes to transcriptional regulation: auxin response factors (ARFs) and auxin protein of early response (Aux/IAA) (Remington et al., 2004). *C. papaya* only has 18 Aux/IAA sequences and 12 ARFs sequences, this reduction in the number of members in comparison to *A. thaliana* sequences may be due to a process of domestication and the evolutionary history that has undergone the *C. papaya* plant, however the processes of regulation during the development of the plant are little known and very little is known about the evolutionary history that has suffered over the years. With the sequences identified of both species, was determined the percentages of identity of CpAux/IAA and CpARFs proteins sequences of *C. papaya* and of their orthologs AtAux/IAA and AtARFs of *A. thaliana*. The high percentages of sequence identity of CpAux/IAA12 with AtAux/IAA12, CpAux/IAA14 with AtAux/IAA14 and CpAux/IAA13 with AtAux/IAA28 is due to their respective signatures that suggest a possible regulatory mechanism of the Aux/IAA family. Members CpARF5, CpARF 6 and CpARF7 showed the N-terminal B3-type DNA binding domain (DBD), a variable middle region that functions as an activation domain (AD) enriched in glutamine (Q) along with leucine (L) and serine (S) residues, a carboxy-terminal dimerization domain (CTD: domains III/IV). These domain III/IV domains are involved in protein–protein interactions by dimerizing with repressor genes of Aux/IAA type that suppress the activity of ARFs in the absence of auxin (Ulmasov et al., 1997; Kim et al., 1997; Guilfoyle and Hagen, 2007; Piya et al., 2014). Likewise the CpARF5, 6, 7 sequences was Q-rich, this condition has been observed in five ARFs proteins of *A. thaliana* (ARF5, ARF6, ARF7, ARF8, ARF19) and were characterized as transcriptional activators based on transient assays in transfected protoplasts involved in root formation (Ulmasov et al., 1999a; Tiwari et al., 2003), therefore we think that CpARF5, CpARF6, CpARF7 genes identified in *C. papaya* also may be involved in root formation.

Our study made it clear that seedlings of *C. papaya*, low expression of auxin repressor genes (CpAux/IAA12, CpAux/IAA13 and CpAux/IAA14) and higher expression of auxin response factor (CpARF5, CpARF6 and CpARF7) in SB and R was observed, therefore in control plants there is no repression of auxin transcription. We think that the high rhizogenic capacity in *C. papaya* plants is due to the rapid degradation of CpAux/IAA12-13-14 through of the protein ubiquitin ligase SCF^{TIR1} activating to CpARF5, 6, 7. For this

reason, is presumed that the endogenous IAA must be transported basipetally (from apical areas towards the apex of the root) and it moves acropetally (from the root apex to apical zones) by means of external layers of the root (Muday and DeLong, 2001) for the root formation in *C. papaya* plants.

In vitro plantlets exposed in treatments without IBA in their culture medium showed high expression of genes that repress the auxin transcription (CpAux/IAA12-13-14), therefore the transcriptional regulation is negative and results in the inhibition of root formation, in contrast, *in vitro* plantlets supplemented with IBA showed high rooting rates and high expression of auxin response factors (CpARF5, 6 and 7) in SB and R tissues. Emphasis in the section previous was observed that CpARF5, CpARF6 and CpARF7 act in an important way in the regulation for lateral root formation. With respect to this affirmation, Zenser et al. (2001) also mention that the application of exogenous auxin in plants increases the rate of degradation of Aux/IAA proteins, leading to an increase in the activity of the ARFs. Likewise, Wilmoth et al. (2005) mention that double mutant plants in ARF19 and ARF7 produce very few lateral roots even in the presence of exogenous auxin and a wild-type plant does not restore the production of its roots when is grafted to a double mutant pattern. Analogously, mutations of *IAA* with function-enhancing, affect the inhibition of auxin root by auxin, thus different Aux/IAA proteins may negatively regulate ARF7 and ARF19 genes in root meristems (Reed, 2001).

3.6. CONCLUSION

It appears that the addition of exogenous auxin causes, on the one hand, a decrease in the expression of the auxin repressor genes (CpAUX/IAA12, CpAUX/IAA13 and CpAUX/IAA14), and on the other hand, it induces the expression of the auxin promoter genes (CpARF5, CpARF6 and CpARF7) on *in vitro* plantlets of *C. papaya*. This is clear from the analysis of our expression results shown in Figure 3.5 and Figure 3.6, when those plantlets that were not exposed to exogenous auxin, had a high expression of the auxin repressor genes at the SB tissue, but very low expression of the auxin promoter genes. On the contrary, those *in vitro* plantlets exposed to the auxin IBA, showed very low expression of those repressor genes, but a high expression of the promoter genes in the resulting roots. It is interesting, that the expression levels of CpARF in the roots of the IBA auxin treated *in vitro* plantlets, almost reached values shown by the roots from the de-rooted seedlings.

It is also interesting, that *in vitro* plantlets from those treatments that induced a high expression of the genes CpARF, were able to induce roots. On the contrary, *in vitro* plantlets from those treatments that resulted in low expression levels of CpARF, but high expression levels of CpAux/IAA, were unable to form roots.

It is noteworthy, the case of the treatment L6, where *in vitro* plantlets despite not being exposed to exogenous auxin, did generate roots solely as a result of being exposed to high light levels in the growth room. It is likely, that in this particular treatment, the elevated light levels might have induced increased concentration of endogenous auxins. More research is required to confirm this possibility.

Other interesting result was the fact that ventilation of the *in vitro* containers consistently resulted in increased expression levels of CpARF, at the time that resulted in increased root numbers and root length, higher rooting percentage and even better survival rates, than their non-ventilated counterparts.

CAPÍTULO IV. DISCUSIÓN GENERAL Y CONCLUSIONES

4.1. DISCUSIÓN GENERAL

Debido a un suministro continuo de sacarosa exógena, nutrientes y condiciones de luz deficientes, las *in vitro* plantúlas presentan un desarrollo deficiente del aparato fotosintético, que provoca una ausencia de la fotosíntesis (Rodriguez et al., 2008). Estas *in vitro* plantas son pobres en clorofila, produciendo así una baja asimilación de carbono. Durante la aclimatación, este mal funcionamiento del aparato fotosintético obliga a las plantas a utilizar las reservas obtenidas durante el cultivo *in vitro* hasta que la producción de sacarosa sea suficiente para el desarrollo normal de la planta (Rodriguez et al., 2008), sin embargo si durante este lapso de tiempo no existe el cambio del metabolismo heterotrófico al autotrófico puede dar como resultado un alto porcentaje de mortandad de las plantas durante la aclimatación a condiciones *ex vitro*.

Como cualquier otra planta, *C. papaya* cuando es cultivada *in vitro*, debe de realizar el cambio al sistema autótrofo para mejorar su aclimatación *ex vitro*. Además, otro problema que presenta esta especie es la baja capacidad de formación de raíces adventicias, por lo anterior, se han utilizado auxinas naturales como el Ácido Indol-3-Acético (AIA) o sintéticas como el Ácido Indol Butírico (IBA) necesarios para promover la iniciación del enraizamiento o emergencia de las raíces adventicas. Los tratamientos rizogénicos han demostrado que las *in vitro* plántulas de *C. papaya* son capaces de formar raíces adventicias únicamente cuando se les suministra IBA en el medio de cultivo. En relación a la produccion de raíz, cuando *in vitro* plántulas son suministradas con una mayor intensidad de luz y adicionadas con IBA exógena, se promueve una mayor producción de raíz en estas *in vitro* plántulas de *C. papaya* cv. Maradol.

De nuestros tratamientos realizados, creemos que el etileno influye en los tratamientos realizados inhibiendo o formando raíces adventicias de *in vitro* plántulas de *C. papaya*, sin embargo, hasta la fecha no queda claro como el etileno promueve la formación de raíces adventicias en algunas especies, mientras que en otras especies las inhibe. La interacción etileno-luz regulan el metabolismo de la auxina durante el crecimiento del hipocótilo (Smalle et al., 1997; Collett et al., 2000), asímismo, hay suficiente evidencia que sugieren

que la interacción etileno-azúcar desempeñan papeles importantes en varios aspectos del desarrollo de las plantas (Gazzarrini and McCourt, 2001; Hartig and Beck, 2006; Eveland and Jackson, 2012).

La sacarosa actua como molécula de señalización y como regulador de la expresión génica (Smeekens et al., 2010), este azúcar podría tener un efecto negativo en el metabolismo de la auxina, desde la biosíntesis hasta el transporte, la percepción y la señalización, se podría conducir en un alterado crecimiento y en el desarrollo anormal de la planta (Mishra et al., 2009). Nuestros tratamientos con bajo contenido de sacarosa no inducen el crecimiento radicular en *in vitro* plántulas de *C. papaya*, sin embargo, a nivel de expresión la glucosa en general puede controlar algunos procesos no transcripcionales tales como la estabilidad y/o degradación de proteínas tipo Aux/IAA o ARFs afectando la señalización y respuestas mediadas por auxina (Mishra et al., 2009).

Asímismo, suponemos que cuando las *in vitro* plántulas fueron suministradas con una mayor intensidad de luz provocó en una mejor fotosíntesis, además de generar una mejor translocación de los carbohidratos a la raíz y del crecimiento y morfología del sistema radicular como fue mencionado previamente por Hermans et al., (2006); Hammond and White, (2011). Nuestros datos sugieren que la interacción luz-auxina actúan de forma sinérgica en el desarrollo de raíces adventicias, sin embargo todavía nos falta por aclarar como diferentes espectros de luz podrían afectar la formación de raíces adventicias. En plantas de *A. thaliana* se ha demostrado que cuando estas son cultivadas a baja intensidad de luz, se reduce la acumulación de la biomasa de las raíces y la longitud total de la raíz (Aresta and Fukai, 1984; Buttery and Stone, 1988; Demotes-Mainard and Pellerin, 1992; Nagel et al., 2006).

El resultado de tener a las plántulas ventiladas expuestas a CO_2 ambiental se manifestó en un mayor crecimiento de las plantas cuando fueron aclimatadas, además estas plantas presentaron raíces más largas. Creemos que estas plántulas ventiladas *in vitro* al ser expuestas a CO_2 ambiental aumentaron su resistencia a la deshidratación, lo que posiblemente la plántula tuvo que pre-acondicionarse para mejorar su fotosíntesis antes de ser expuestas a condiciones *ex vitro*. Reportes previos han demostrado que un alto CO_2 aumenta el crecimiento de las plantas cultivadas *in vitro* incluso bajo estrés hídrico

(Wall et al., 2006). Asímismo, se ha informado que el aumento del crecimiento de las plántulas es una consecuencia del aumento de la tasa fotosintética, debido al control de las condiciones ambientales (Hahn and Paek, 2001; Shin et al., 2014). En nuestros resultados las plantas no ventiladas necesitaban más tiempo para aclimatarse a las condiciones ambientales; estas plantas presentaron alta mortandad. Adicionalmente está técnica con sistemas ventilados ha sido utilizado frecuentemente por micropropagadores para la obtención de clones con mejores porcentajes de sobrevivencia.

Posteriormente conociendo cuales son los genes reguladores que actúan en la transcripción (genes represores: Aux/IAA12, Aux/IAA14, Aux/IAA28 y genes activadores: ARF5, ARF6, ARF7. ARF8, ARF19) y el transporte polar de auxinas (AUX1, LAX1, LAX2, LAX3 y PIN1, PIN2, PIN3, PIN4, PIN5, PIN6, PIN7 PIN8) necesarios para la formación de raíces laterales en *A. thaliana* (De Smet et al., 2010), se procedió a identificar sus respectivos genes ortólogos en *C. papaya* los cuales aún eran desconocidos. Por lo anterior, en la presente tesis se caracterizó la estructura y filogénia de genes ortólogos tipo CpAux/IAA-CpARFs y CpAUX1/LAXs-CpPINs en el genoma secuenciado de *Carica papaya* L. cv. Sun Up. Adicionalmente, con la ayuda de porcentajes de identidad y de análisis filogenéticos se identificó en el genoma de *C. papaya* secuencias ortólogas de genes represores de tipo CpAux/IAA12, CpAux/IAA13, CpAux/IAA14 y de genes activadores CpARF5, CpARF6, CpARF7, involucrados en reprimir y activar, respectivamente la transcripción de auxina para la formación de raíces. Asímismo, se identificó la familia completa de genes involucrados en el transporte polar de flujo de entrada (CpAUX1, CpLAX1, CpLAX2, CpLAX3) y de salida (CpPIN1, CpPIN2, CpPIN3, CpPIN4, CpPIN5, CpPIN6) de la auxina.

Aunque aún falta más pruebas para corroborar nuestros datos, los resultados del presente estudio sugieren que plantas de semilla, plantas desprovistas de su raíz e *in vitro* plántulas de *C. papaya* únicamente son capaces de producir raíces adventicias cuando están expuestas a condiciones que favorezcan una alta expresión de genes activadores de auxinas (CpARF5, CpARF6 y CpARF7) y de genes involucrados en el transporte polar de auxinas (CpAUX1, CpLAX1-3 y CpPIN1-6), mientras que estas plantas presentaron una baja expresión de genes represores (CpAux/IAA12, CpAux/ AA13 y CpAux/ AA14) (Tabla 4.1). Por el contrario, las plántulas *in vitro* que no producen raíces (en ausencia de

auxina exógena) presentan una alta expresión de genes represores y una baja expresión de activadores y de genes involucrados en el transporte polar de auxinas (Tabla 4.1).

Suponemos que la adición exógena de IBA provoca tal vez un incremento en la concentración de auxinas endógenas en los tejidos, ocasionando la degradación (vía ubiquitinación) del proteosoma 26S de proteínas CpAux/IAA permitiendo que proteínas CpARFs promuevan la transcripción y por consiguiente un adecuado transporte polar de auxina durante la formación de la raíz (rizogénesis) en *in vitro* plántulas de *C. papaya* cv. Maradol.

4.2. CONCLUSIONES

- Se diseñaron diferentes tratamientos que inducen la formación de raíces adventicas en *in vitro* plántulas de *Carica papaya* L. cv. Maradol (especie con baja capacidad rizogénica cuando es cultivada *in vitro*).

- Sin la adición de Ácido Indol Butírico (IBA) en el medio de cultivo, las *in vitro* plántulas de *C. papaya* cv. Maradol no son capaces de formar raíces adventicias.

- *In vitro* plántulas de *C. papaya* cv. Maradol presentan altos porcentajes de enraizamiento y sobrevivencia (superior al 80 %) cuando se les suministra 2 mg L^{-1} de IBA en el medio de cultivo, se aumenta la intensidad lumínica (750 µmol fotón m^{-2} s^{-1}) y/o son crecidas en un adecuado sistema de ventilación que evite la acumulación de etileno que pudiera inhibir la formación de las raíces adventicias.

- Se identificaron *in sílico* en el genoma de papaya transgénica *(Carica papaya* L.*)* cv. Sun Up, 12 genes reguladores de la transcripción de auxina que modulan la formación de raíces (CpARF5, CpARF6, CpARF7) y 18 genes de respuesta temprana de auxina (CpAux/IAA12, CpAux/IAA13 y CpAux/IAA14) encargados de dimerizar con genes ARFs reprimiendo su actividad y por consiguiente inhibiendo la formación de raíces.

- Se caracterizó *in sílico* en el genoma de papaya transgénica *(Carica papaya* L.*)* cv. Sun Up*,* la estructura y filogenia de la familia completa de 4 genes involucrados en el transporte polar de auxina (PAT) de flujo de entrada (CpAUX1, CpLAX1, CpLAX2 y CpLAX3) y 6 de salida (CpPIN1, CpPIN2, CpPIN3, CpPIN4, CpPIN5 y CpPIN6).

- Plantas de *Carica papaya* L. cv. Maradol provenientes de semilla (plantas control) las cuales no muestran problemas de enraizamiento, presentan en tejido de la base del tallo y raíz una baja expresión de genes represores de la transcripción de auxina (CpAux/IAA12, CpAux/IAA13 y CpAux/IAA14), pero una alta expresión de

genes activadores de la transcripción de auxina (CpARF5, CpARF6 y CpARF7). Adicionalmente, dichas plantas presentan una alta expresión de todos los genes involucrados en PAT de flujo de entrada (CpAUX1, CpLAX1-3) y de salida (CpPIN1-6) de la auxina.

- En el caso de las *in vitro* plántulas de *C. papaya* cv. Maradol que no son capaces de formar raíces adventicias (sin adición de IBA en su medio de cultivo) presentan en tejido de la base del tallo y raíz una alta expresión de genes que reprimen la transcripción de auxinas (CpAux/IAA12, CpAux/IAA13 y CpAux/IAA14) pero una baja expresión de genes activadores de la transcripción y de genes involucrados en el transporte polar de auxina.

- Por el contrario, *in vitro* plántulas de *C. papaya* cv. Maradol capaces de formar raíces adventicias (con adición de IBA en su medio de cultivo), presentan en tejido de la base del tallo y raíz una alta expresión de genes activadores de la transcripción de auxina (CpARF5, CpARF6 y CpARF7) y de genes involucrados en el transporte polar de auxina (CpAUX1/LAX1-3 y CpPIN1-6). Estas *in vitro* plántulas no presentan una represión de la transcripción de la auxina porque presentaron baja expresión de los genes CpAux/IAA12, CpAux/IAA13 y CpAux/IAA14.

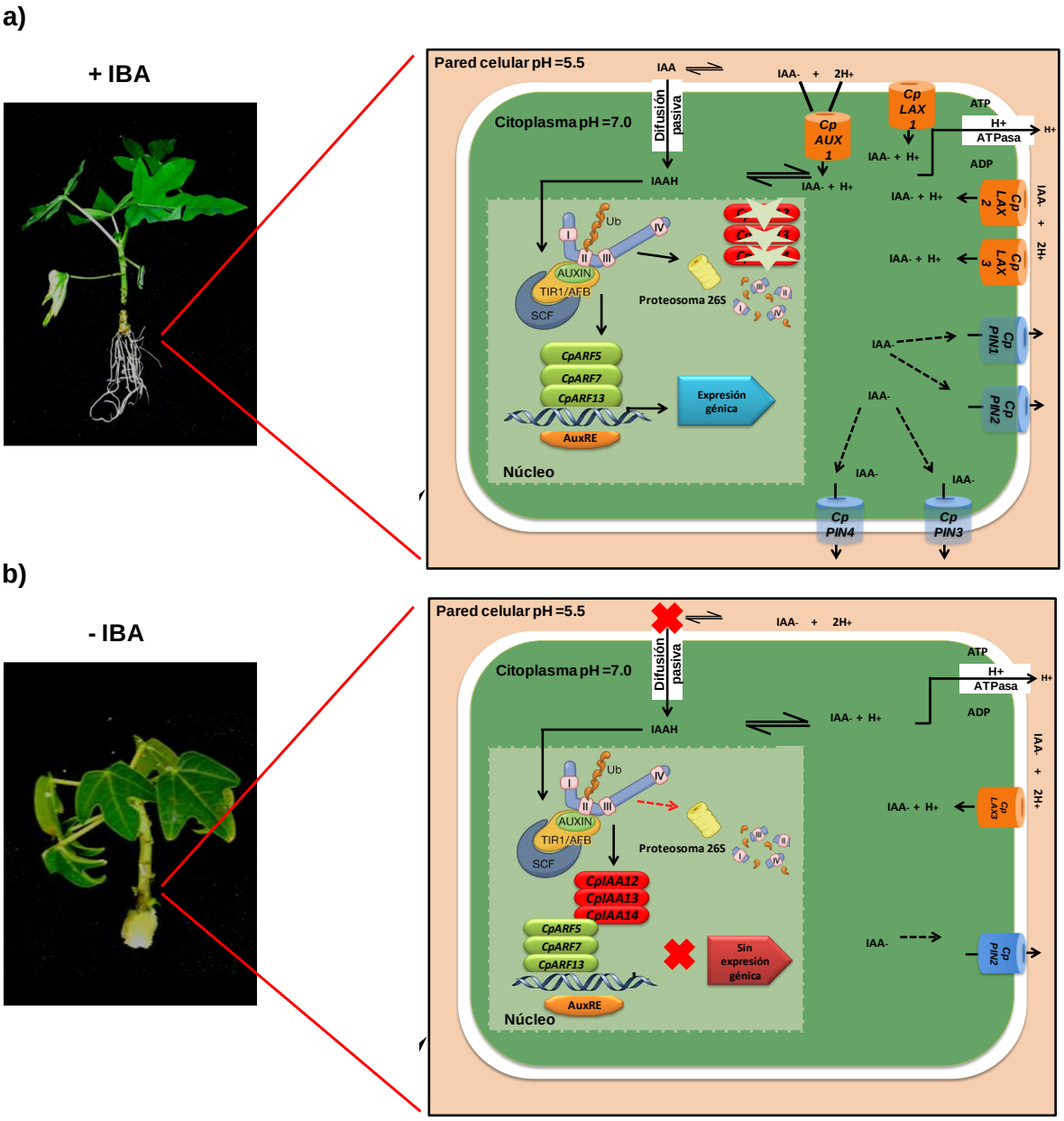

Figura 4.1. Modelo explicativo de la regulación transcripcional y del transporte polar de auxina en raíces adventicias de *in vitro* plántulas de *C. papaya* cv. Maradol. **(a)** *in vitro* plántulas adicionadas con auxina exógena son capaces de formar raíces adventicias y muestran baja expresión de genes represores (CpAux/IAA12-14) y alta expresión de genes activadores (CpARF5-7) y de genes PAT (CpAUX1/LAX1-3 y CpPIN1-6). **(b)** *in vitro* plántulas sin auxina exógena no forman raíces adventicias y muestran alta expresión de genes represores que conlleva en una baja expresión de genes activadores y de genes PAT.

4.3. PERSPECTIVAS

- Realizar ensayos con NaCl en el medio de cultivo donde las plántulas crecerán y determinar mediante conductancia hidráulica la funcionalidad de las raíces adventicias producidas en las *in vitro* plántulas de *C. papaya* cv. Maradol.

- Cuantificar el contenido de auxina endógena en *in vitro* plántulas de *C. papaya* cv. Maradol expuestas en los diferentes tratamientos inductores de raíces adventicias.

- Mediante la técnica de amplificación rápida de los fragmentos terminales del ADNc (RACE) se pretende mapear los extremos terminales 5' y 3' de las ORF predichas de las secuencias de CpARF5, CpARF6 y CpARF7, así mismo se podrá comprobar que los marcos de lectura encontrados *in silico* de estos genes corresponden a los marcos de lectura reales clonados *in vitro* dentro del transcriptoma de *C. papaya* cv. Maradol.

- Dentro de la familia de los factores de transcripción de auxina (ARF) se encuentran genes específicos que regulan la formación de raíces laterales en *A. thaliana* (ARF5, ARF6 y ARF7), por tanto, al sobreexpresar genes ortólogos (CpARF5, CpARF6 y CpARF7) previamente clonados mediante RACE en el transcriptoma de *C. papaya* cv. Maradol se podrán obtener plantas de papaya que presenten mejores sistemas radiculares.

- Confirmar mediante parámetros fisiológicos-morfológicos el aumento de las raíces adventicias en *in vitro* plántulas de *C. papaya* cv. Maradol las cuales han sido sobreexpresadas con genes CpARF5, CpARF6 y CpARF7.

- Cuantificar la sobreexpresión mediante RT-qPCR de los genes CpARF5, CpARF6 y CpARF7 en las *in vitro* plántulas regeneradas de *C. papaya* cv. Maradol.

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